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Acute Salmonella infection in swine

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Introduction

Salmonella are facultative anaerobic gram-negative bacilli shaped bacteria of the family *Enterobacteriaceae*. The *Salmonella* genus consists of two species: *bongeri* and *enterica*. *Salmonella* are ubiquitous in the environment, and are commonly found in the digestive tract of warm and cold-blooded animals (56, 99, 140, 144). Over 2,500 serovars of *Salmonella* are recognized based on the White-Kauffmann-Le Minor scheme, which identifies serovars based on their somatic, capsular, and flagellar antigens (69). Most serovars are motile and contain peritrichous flagella, excluding non-motile *S.* serovar Gallinarum and other variably motile and non-motile flagellar mutants (56, 144).

Salmonella sp. produce specific biochemical reactions that help differentiate the genus from other bacteria. These tests include positive reactions for oxidase, catalase, methyl red, Simmons citrate, lysine decarboxylase, and ornithine decarboxylase (56). The genus characteristically produces negative test results for indole, Voges-Proskauer, and urea (56). Hydrogen sulfide is produced by most but not all *Salmonella* serovars (56).

Salmonella are facultative intracellular pathogens capable of causing disease in a broad range of hosts including humans and animals. More than 2,500 serovars have been identified, and most of them are thought to be capable of causing disease (144). The predilection of this organism for the intestinal tract of food-producing animals allows the organism to readily contaminate the environment, water supplies, and food products (5, 63, 88, 96).

Approximately 1.4 million cases of human salmonellosis occur annually in the United States (9.7% of all foodborne illnesses), resulting in over 15,000 hospitalizations and over 500 deaths (30.6% of all foodborne deaths) (42, 90). Of these cases it is estimated that 95% occur due to foodborne transmission of contaminated food products (42, 90). Pork, poultry, beef, and egg products continue to be important sources of human salmonellosis (31, 88, 96, 110, 113). *Salmonella* are capable of entering the human food chain by many different modes.

Salmonella can infect and establish in young pigs creating a carrier or latent infection state in which pigs periodically shed *Salmonella* throughout their lives (50, 142, 143). Shedding by infected animals on the farm, in transportation vehicles, and at slaughter can perpetuate *Salmonella* infection (36, 50, 61, 62, 94, 127, 142, 143). Current slaughter practices in the United States recommends holding swine in lairage prior to slaughter to increase meat quality (100). Pigs from multiple farms of unknown *Salmonella* history are frequently co-mingled together in lairage holding pens. A majority of these holding pens have been shown to be highly contaminated with *Salmonella* (112). During this period animals may become infected with *Salmonella* by consumption of contaminated feces and water while in lairage (36, 60-62, 94). *Salmonella* can survive and spread to pork carcasses during processing if not removed by superficial carcass cleaning methods, such as scalding, acidification, or fire singeing processes (125, 126). Additionally, *Salmonella* may escape the gastrointestinal tract and contaminate meat during processing, including the disembowelment and de-bunging processes (20, 124-126). Finally, *Salmonella* may enter the food chain during preparation and handling immediately prior to consumption.

During the 1990s in the U.S. concern regarding *Salmonella* contamination of pigs and meat products arose when, during epidemiological studies into identification of slaughterhouse critical control points, it was identified that pigs free of *Salmonella* were becoming acutely infected with *Salmonella* during the periods of transport and lairage (61, 62, 73). This acute *Salmonella* infection had been previously reported in experimental models in both cattle and swine (28, 39). More specifically acute infection can be defined as a rapid infection that can occur within hours after inoculation and disseminate to both alimentary and non-alimentary tissues (39, 61, 83). These studies determined that *Salmonella enterica* subspecies *enterica* serovar Typhimurium were capable of acutely infecting and rapidly disseminating to both non-alimentary and alimentary tissues in esophagostomized animals (28, 39). These papers proposed that *Salmonella* may be able to enter a host through a novel unstudied route and mechanism (39).

Swine Salmonellosis

In the U.S. it is estimated that millions of dollars in production losses occur each year due to *Salmonella* infection and disease in pigs (117). Infected animals are thought to be the most important source of *Salmonella* to other animals (139). *Salmonella* are frequently isolated from the gastrointestinal tract of clinically healthy carrier animals, which can intermittently shed and expose *Salmonella* to susceptible animals (139). Salmonellosis is commonly found to affect animals in units with high pig densities, poor sanitation, immunological naïve populations, or as a sequel to other infections (30, 95, 104, 121, 139).

Host adapted *S.* Choleraesuis and non-host adapted *S.* Typhimurium are two of the most frequent isolated serovars recovered from clinically diseased swine (127). Excluding *S.* Derby, *S.* Agona, and *S.* Heidelberg, which are also commonly associated with clinical

disease in swine, other non-host adapted serovars can cause transient clinical disease but are not frequently isolated from clinically ill pigs (35, 127). *S.* Choleraesuis causes a severe septicemic disease, while infection with non-host adapted *Salmonella* generally results in enterocolitis (105, 139).

S. Choleraesuis can cause disease in pigs at all stages of production, but primarily affects weaned pigs younger than 5 months of age (10, 139). Morbidity generally is below 10%, but mortality rates can be quite high. Systemic salmonellosis can manifest as pneumonia, hepatitis, enterocolitis, and less frequently abortion and meningoencephalitis (10, 139).

Clinical signs of animals with acute *S.* Choleraesuis salmonellosis include: pyrexia, anorexia, respiratory distress, and lethargy (10, 105, 139). Animals may be found dead or huddled together not wanting to move. Diarrhea is not usually associated with the disease until 3 or 4 days post-infection. Duration of the disease is dependent on the speed of diagnosis and intervention of disease transmission.

Gross pathology of septicemic salmonellosis can include: dermal cyanosis, gastric mucosal infarction, splenomegaly, hepatomegaly and necrosis, mesenteric lymphadenopathy, and occasionally intestinal necrosis (10, 139). Areas of necrotic intestine can develop into the characteristic "button ulcer" appearance (139). The lungs may exhibit a diffuse interstitial pneumonia with occasional cranioventral bronchopneumonia (10, 139). Petechial hemorrhages may be present on the epicardium and in the renal cortices (10, 139).

Histopathologic lesions may be evident in the liver, stomach, skin, kidneys, and lungs. Multifocal histiocyte aggregation frequently occurs in areas of coagulative heptocellular necrosis and diffusely throughout the interstitium of the lung (10, 139).

Fibrinoid thrombi can be seen in the vessels of the stomach, skin, kidneys, and occasionally lungs (10, 139).

Clinical signs of septicemic salmonellosis can be difficult to differentiate from other systemic diseases. Diagnosis of *S.* Choleraesuis infection requires the isolation of *Salmonella* recovered in significant numbers from tissues from acutely ill animals (139). Isolation of *Salmonella* from animals receiving antimicrobial treatment may not allow for proper isolation and diagnosis, thus perpetuating the disease in the herd.

Many non-host adapted *Salmonella* are capable of infecting and causing enterocolitis in pigs (127). *S.* Typhimurium, Choleraesuis, and Heidelberg are the most common isolates from clinically affected animals (35, 127). *Salmonella*-induced enterocolitis commonly affects animals from weaning to 4 months of age and can quickly spread between animals housed together (10, 139).

Enterocolitis by non-host adapted *Salmonella* is characterized by watery yellow diarrhea that may or may not contain mucus or blood. Diarrhea may persist for 3 to 7 days and recur for several weeks. Animals are pyretic, anorectic, and usually dehydrated due to the loss of water and electrolytes in the diarrhea. The majority of infected animals recover though some may not completely eliminate the organism, inducing the carrier state.

Gross pathology in animals displaying acute disease due to non-host adapted *Salmonella* includes areas of necrotic small intestine, cecum, and/or colon (10, 105, 139). Mesenteric lymph nodes of infected animals are usually enlarged and edematous.

Microscopic pathology is characterized by areas of necrosis and leukocyte infiltration. Commonly, the small intestinal crypts, cecal glands, colonic glands and enterocytes are focally to multifocally necrotic and contain varying amounts of inflammatory

cells (10, 105, 139). In early infection neutrophils are found in areas of invasion, whereas in more chronic disease, macrophages and lymphocytes are the prominent leukocytes found (150). During acute disease Peyer's Patches may become necrotic, but during chronic infection Peyer's Patch hypertrophy is more common.

Virulence factors

Salmonella contain many different virulence factors that aid in their colonization and the development of disease in humans and animals. Non-specific virulence factors include lipopolysaccharide (LPS), fimbrae, flagella, and in some serovars capsules (2, 43, 80, 133, 138). LPS, also known as endotoxin, is capable of inducing the production of cytokines and inflammatory responses, activating the coagulation cascade, and can ultimately induce endotoxic shock and disseminated intravascular coagulopathy (16, 32, 93). The LPS of *Salmonella* has also been shown to activate the alternative complement pathway, increasing the uptake of this facultative intracellular pathogen into mononuclear leukocytes (78). LPS, flagella, and fimbrae aid in colonization and development of disease by facilitating movement in the host and attachment to host gastrointestinal epithelium (2, 79, 138). Other virulence determinates specifically associated with *S.* Dublin, and *S.* Typhi include the production of capsules that aid in evading the hosts immune system by reducing phagocytosis (129).

Salmonella contain specific virulence factors, which include the production of toxins and other virulence determinates, that are encoded in chromosome associated *Salmonella* pathogenicity islands (SPI) (12, 52). SPI-1 encodes for a type III secretion system that is used to insert *Salmonella* proteins into host cells (12, 52). These translocated proteins interfere in cell signal transduction and membrane ruffling which allows *Salmonella* to enter

non-phagocytic cells and induce apoptosis of phagocytic cells (52). SPI-2 encodes for a type III secretion system that inserts *Salmonella* proteins into the host, and aids in the ability of *Salmonella* to grow within mammalian cells and tissues (12, 52). SPI-3 encodes for a magnesium transporter that is required for virulence and survival in macrophages (52). SPI-4 and SPI-5 play important roles in toxin secretion and reduction of host inflammatory responses, respectively (52).

In addition to SPI, other parts of the *Salmonella* genome, such as virulence plasmids also encode for virulence factors (52, 54). Most pathogenic *Salmonella* contain a virulence plasmid that encodes for spvRABCD genes, which have been shown to cause lethal infections by encoding for survival within monocytes, fimbrial proteins, and transcriptional factors (52). Other genes code for antibiotic resistance that can complicate the treatment of clinical disease, but are not directly associated with *Salmonella* virulence (1, 53).

Epidemiology and transmission of *Salmonella* in swine

Salmonella enterica subspecies *enterica* serovars are capable of infecting cold- and warm-blooded animals, including swine. The incidence of different *Salmonella* serovars in swine populations tends to vary between countries or even between different regions of a country (7, 8, 35, 87). This reported variation is most likely due to evolutionary differences between serotypes in adapting to special ecological niches (52, 53, 71, 144). Additionally, these variations help to emphasize the importance of national monitoring programs for the incidence of *Salmonella* in respective swine populations.

Salmonella in pigs pose three primary problems: disease, carrier states, and contamination of the food supply. Both host adapted *S.* Choleraesuis and non-host adapted serovars can cause disease in swine. *S.* Choleraesuis, Typhimurium, Derby, and Heidelberg

are the serovars most frequently isolated during clinical outbreaks in pigs (127). A number of animals recovering from clinical infections of *Salmonella* are capable of harboring the organisms, though clinical disease is no longer evident. These animals are termed carrier animals.

Salmonella carriers may harbor *Salmonella* in the tonsils, gastrointestinal tract, or mesenteric lymph nodes (142, 143). Because these organisms are not predictably passed in the feces, negative fecal cultures can result (139). Experimental models of *Salmonella* carriers in swine exist but may differ from natural carrier states depending on the route and dose of exposure (49, 51). Studies are frequently conducted to determine the prevalence of *Salmonella* on U.S. farms, and to study the dynamics of *Salmonella* on farms, in pigs, and in pork products over time (24, 35, 46, 72, 81, 132). The National Animal Health Monitoring System (NAHMS) indicated that in 1995, *Salmonella* was recovered in 38.2% of the finishing herds in the U.S. (127). *Salmonella* have been isolated from all stages of swine production (45).

Carrier animals are frequently found to shed latent *Salmonella* during periods of weaning, environment changes, food withdrawal, transportation, and co-mingling with unfamiliar pigs (4, 50, 65, 142, 143). *Salmonella* are susceptible to heat, sunlight, and most disinfectants, but are fairly resistant to drying and freezing (58). These characteristics allow *Salmonella* to survive for days to years in water, soil, wet fecal slurries, and dry fecal matter (48). Carriers may intermittently shed *Salmonella* into their environment for weeks to months causing periodic outbreaks and spreading the organism on the farm or immediately prior slaughter (48, 49, 51, 62, 139, 143).

Salmonella can acutely infect pigs during lairage and transportation prior to slaughter (60-62). Studies have shown that *Salmonella*-free pigs, as tested on their farms of origin, are capable of acquiring and becoming infected with *Salmonella* within a short period of time during transport and lairage. Rapid *Salmonella* contamination may be due to stress-induced shedding by latently infected animals or due to the rapid infection of clean animals by *Salmonella* shedders during co-mingling at the abattoir prior to slaughter. Contamination of clean animals from *Salmonella*-positive herds is in part due to the length of time in lairage and to the ease in which natural *Salmonella* transmission can occur from environmental contamination, nose to nose transmission, or airborne transmission (60, 102). Though length of time in lairage has been shown to be a critical control point for *Salmonella* contamination, little has been done to shorten this time due to improved meat quality in animals that are held at least two hours prior to slaughter (94, 100, 124).

Acute infection in swine was first demonstrated when virulent *S.* Typhimurium strain χ4232 were isolated from intestinal and non-intestinally associated tissues three hours after intranasal challenge in esophagostomized pigs (39). This suggested that an alternative route of *Salmonella* invasion may exist in which *Salmonella* can rapidly infect internal organs and the alimentary system by means other than entering through the intestinal tract.

Immune responses to *Salmonella* in swine

Host defenses to *Salmonella* can be broadly classified into two categories: innate immunity and adaptive immunity (40, 146). Pigs produce a number of innate non-specific immune responses to many fecal-oral pathogens. Stomach acids keep the pH of the stomach suboptimal which impedes the growth of *Salmonella* (41, 139, 144). Host derived proteins, such as defensins and proteolytic enzymes, form pores and erode outer bacterial membranes,

which can cause bacterial cells to lyse (66, 130). The natural microflora is capable of producing acids which lower luminal acidity, oxygen radicals, and bactericins that act on bacterial cell membranes and prevents pathogens from binding to host receptors (37). Non-specific host immune cells, such as natural killer cells and γδ T cells, can quickly respond to foreign antigens, alerting the host to their presence (66, 130, 146).

Salmonella are capable of evoking strong adaptive immune responses. Though adaptive immune response are capable of preventing disease and eliminating the organisms, approximately 10 to 14 days are required following initial infection to generate an effective immune response against *Salmonella* sp. By convention, the somatic antigen, located on the outer portion of the lipopolysaccharide, is capable of activating macrophages which stimulate T helper 2 cells that aid in the initiation of a humoral response (66, 130). Antibodies that form with an affinity to the somatic antigen aid in blocking host specific bacterial receptors, in opsonization, and in the activation of the alternative complement pathway (66, 130). The ability of *Salmonella* to survive in host cells allows the organism to evade a portion of the host's humoral immune response (32, 52, 57, 67, 145). In order to eliminate invading *Salmonella*, an effective humoral and cell mediated immune response must be initiated (25, 57, 70). *Salmonella* that are phagocytosed by host macrophages also elicit a T helper 1 response that results in activation of macrophages that aid in bacterial killing (25, 57, 70). Attenuated live *Salmonella* vaccines that manipulate the activation of the adaptive immune responses have been developed which quickly activate host immune responses upon recognition of invading *Salmonella* and are efficacious in reducing clinical salmonellosis (11, 22, 25, 109, 122).

Methods of detection

Salmonella can be detected both directly and indirectly by a number of different methods. Culture and subsequent biochemical testing is considered to be the gold standard for *Salmonella* identification (6, 26, 27). *Salmonella* can be indirectly detected by agar immunodiffusion tests, immunohistochemistry, and enzyme linked immunosorbant assays (ELISA), such as the Danish mix-ELISA, which use host derived antibodies produced to *Salmonella* antigens (98, 114, 136). *Salmonella*-specific deoxyribonucleic based tests, such polymerase chain reaction, gel electrophoresis, and southern blot hybridization techniques can also be used to identify *Salmonella* (23, 33, 84, 120, 141). *Salmonella* culture and ELISA's are the most reliable and frequently used diagnostic tests currently in use.

Intervention strategies

Many different methods and ideas have been explored in the attempt to reduce economic loss and foodborne illness due to *Salmonella* infection. These methods can be broadly placed into three intervention categories: animal, on-farm, and food.

Animal level intervention methods have focused on the prevention of *Salmonella* infection, control of disease, and elimination of carrier animals, and include the use of antimicrobials and vaccines, chemicals, diet manipulations, the feeding of pre- and probiotics, and phage therapy (3, 11, 18, 22, 29, 59, 75-77, 92, 116, 122, 128, 134).

Killed and modified live *Salmonella* vaccines have been used in attempt to control host specific and non-host specific *Salmonella* isolates in pigs (11, 22, 122). The use of vaccines against host specific *Salmonella* Choleraesuis have greatly reduced the clinical importance of *S.* Choleraesuis to the swine industry (22, 119). The most effective *S.* Choleraesuis vaccines are generally attenuated live vaccines due to the fact that killed

vaccines lack the ability to produce a strong cell-mediated immune response, which is needed to eliminate intracellular *Salmonella* (11, 119) Interestingly, host specific *Salmonella* Choleraesuis vaccines have been shown to provide partial cross protection against non-host specific *Salmonella* sp. in natural infection situations (22, 34, 119). This cross protection in chronic infection models prompted Erdman *et al.* to analyze the ability of commercially available *Salmonella* vaccine strains and other potential *Salmonella* vaccines to reduce acute *Salmonella* infection in swine, but to date results in acute infection models have remained inconsistent (34).

A number of different dietary manipulations to reduce *Salmonella* infections in pigs have been investigated. Common diet manipulations revolve around the fact that low pH environments inhibit *Salmonella* growth (41, 144). Naturally fermented foodstuffs in human diets have lead to the utilization of similar principles in pig diets (91, 134). The use of naturally acidic fermented liquid feed, such as whey or feeds with organic acids added, have been used for many years to increase piglet performance and reduce the prevalence of disease (68, 134, 135, 148). More recently, the use of coarsely ground diets instead of pelleted diets has also been shown to reduce the numbers of *Salmonella* harbored in the gastrointestinal tracts of pigs (92). Though the exact mechanisms by which coarsely ground feeds are successful in inhibiting growth have not been determined, it is thought that the larger particle size of the feed increases the digestibility of the feed by the natural microflora, which in turn increases the release of fatty acids into the gastrointestinal environment and curbs the growth of *Salmonella* (92). Other less profitable and/or less successful diet manipulations have included adding pathogen specific immunoglobins, dried plasma protein, and cytokines to

pig diets in an attempt to neutralize the pathogen and inhibit infection (32, 47, 131). Manipulation of diet to control acute *Salmonella* infection has not been attempted.

In addition to diet manipulation, the use of pre- and probiotics have been attempted to alter the gastrointestinal environment (44, 97). Prebiotics are chemical compounds, given orally, that aid the growth of specific bacterial species in the gastrointestinal tract (44, 97). Prebiotics are specifically developed to aid in the multiplication of lactic acid bacteria (LAB) (44, 97). Probiotics are a mixture of non-pathogenic bacterial (frequently LAB) and/or fungal cultures that aid in gastrointestinal health (44, 97). In principle, probiotics provide beneficial effects by multiple mechanisms. Probiotics can bind to mucosal-associated pathogen gastrointestinal receptors, by binding to the pathogen and sterically hinder binding to the gastrointestinal receptor, produce antimicrobial compounds, produce inhibitory by-products (such as hydrogen peroxide and fatty acids), stimulate the immune system, and aid in growth promotion (44, 97, 106). Selecting probiotics that provide benefit to the host remain a primary concern (44, 97). The use of probiotics to reduce acute *Salmonella* infection has been attempted by the author. The use of single probiotic organisms and probiotic cocktails showed partial reduction in acute infection in gnotobiotic pigs, but had no effect in conventional animals (82).

Prior to 1940 and the discovery of antibiotics, research into and the use of bacteriophage therapy was popular (74). Due to increased concerns of antibiotic resistance, bacteriophage therapy is once again gaining popularity. Bacteriophages are viruses that infect bacteria and specifically infect certain bacterial genera due to bacterial cell surface receptors (101). Once a bacteriophage infects a bacterium the virus may either enter lysogenic or lytic life cycles (101). During lysogeny the bacteriophage lies dormant in the

bacterial host cell by incorporating itself into the host genome. Following infection, during the lytic lifecycle, the bacteriophage takes control of the bacterial proteins and prepares for viral replication (101). Viral particles collect in the bacterial cell until the cell eventually lyses, releasing numerous infectious virions into the surrounding environment (101). Bacteriophage therapy focuses on selecting lytic bacteriophage that kill the bacterial host. The use of bacteriophage to control *Salmonella* in both chronic and acute infection models has been studied (13, 74, 75). In acute infection models bacteriophages were capable of significantly reducing the number of *Salmonella* in the tonsil and cecum of pigs (13, 74). Even though the levels of *Salmonella* were reduced it is believed that reduction was not low enough to reduce the importance of acute *Salmonella* infection during lairage situations (74).

On the farm, intervention strategies to control *Salmonella* can include management techniques, biosecurity, and farm monitoring. Management tools that can influence the number of pigs infected with *Salmonella* and the level of *Salmonella* on the farm include weaning age and age grouping (all-in all-out) practices. Sows may inoculate piglets with *Salmonella* prior to weaning. Weaning piglets at younger ages (6 to 15 days) reduces the likely hood of transmitting *Salmonella* from sow to piglet (55). This practice is not commonly used as it can have negative impacts on the sow, and it reduces piglet weight gain and immunity compared to piglets reared on the sow for longer periods of time. For this reason piglets are commonly weaned at 3 to 4 weeks of age, despite the potential of acquiring *Salmonella*. All-in all-out practices also lower the transmission of *Salmonella* by keeping piglets of similar ages together (reducing the transmission of *Salmonella* from older animals to younger animals) and by regular cleaning of the rooms (reducing the possibility of susceptible pigs from coming into contact with contaminated feces).

Strict biosecurity can also prevent or reduce the amount of *Salmonella* on a farm (45). The ability of *Salmonella* to infect both warm- and cold-blooded species requires that biosecurity measures be taken to keep domestic and wild animals and birds out of pig rearing systems (15, 123). Of particular concern is rodent and bird populations, both of which frequently seek shelter and food during winter months in pig barns (45). Rodents and birds can contaminate pig feed with *Salmonella* allowing for the transmission of the organism to pigs (9, 132). Other biosecurity measures that should be implemented are reducing the number of humans allowed into pig facilities, utilizing and maintaining waste gutter systems, feeding *Salmonella*-free diets, reducing pig densities, controlling temperature variance, and maintaining a animal ward for ill animals that may have a impaired immune systems (45, 55).

Herd monitoring, separate transportation and lairage, and separate slaughter of herds with varying *Salmonella* prevalence levels has been shown to be effective in reducing the levels of *Salmonella* in acute infection models (17, 103, 137). In Denmark, *Salmonella* control programs exist in which farms are monitored monthly and categorized into groups of low, medium, or high *Salmonella* prevalence based on serology and culture (24). Medium and high prevalence farms are monetarily penalized if *Salmonella* levels are found to be excessive. This program mandates the practice of separate transport, lairage, and slaughter of animals from farms with low *Salmonella* prevalence from those with high and medium *Salmonella* prevalence. Additionally, in lairage animal pens are washed between groups in order to physically remove feces that may harbor foodborne pathogens. Interestingly, this separation and routine washing between differing prevalence levels and groups has decreased the incidence of *Salmonella* isolation from pig cecal contents and slaughter carcasses from

these low prevalence farms (17, 103). This routine *Salmonella* monitoring and cleaning of animal pens may prove sufficient to reduce acute *Salmonella* infection. Preliminary studies in the U.S have shown that disinfection alone does little to reduce the number of *Salmonella* isolated in pigs following slaughter (115). The discrepancy in Danish and U.S. results may be due to the fact that U.S. slaughter plants lack segregation of pigs with different *Salmonella* prevalence during lairage. The failure to separate these groups may have allowed animals from low prevalence herds to acquire *Salmonella* from higher prevalence pigs during co-mingling under lairage conditions.

New processing procedures and techniques have been studied and implemented to reduce a variety of foodborne disease agents, including *Salmonella,* during slaughter and meat processing. Hazard analysis and critical control points (HACCP) have been established to monitor and reduce the amount of contamination at the slaughter house and during packaging (19, 86). Other methods to control contamination and cross-contamination of meat during slaughter and packaging include the use of dehairing equipment, hot water treatments, and organic acid washes on carcasses (14, 118, 147). Cooking meat to temperatures above 160° F may be the most simple and economical method to reduce foodborne pathogens prior to consumption, but is infrequently preformed due to taste alterations. Other methods to limit foodborne pathogens that reach consumers include the addition or use of organic acids, bacteriocins, irradiation, preservative salts, meat smoking, and fermentation (21, 38, 64, 85, 89, 107, 108, 111, 149).

The objectives of this dissertation are focused on defining acute *Salmonella* infection in pigs. More specifically, the objectives are to determine if *Salmonella* serovars, besides the previously studied abilities of *S.* Typhimurium, are capable of causing acute infection; to

determine if varying *Salmonella* serovars acutely infect pigs to different degrees; to determine the minimum infectious dose required to cause acute *Salmonella* infection; and to evaluate probiotics in a germfree model of acute *Salmonella* infection.

Dissertation Organization

This dissertation contains four papers previously published in, accepted for publication, or prepared for submission to scientific journals. The author of this dissertation is the primary author of each paper; the co-authors aided in the respective studies by providing technical skills, providing bacterial strains or germfree diets for evaluation, providing advice on study organization and designs, or by providing manuscript review prior to publication. Tables referred to in each chapter are included at the end of the chapter. Following the last paper is a general conclusions chapter and appendices, which comprise an additional journal manuscript and a patent disclosure. A reference section is included at the end of each chapter.

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CHAPTER 2: ACUTE INFECTION OF SWINE BY VARIOUS *SALMONELLA* SEROVARS

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A. T. Loynachan, J. M. Nugent, M. M. Erdman, and D. L. Harris

Introduction

Salmonella are ubiquitous organisms capable of causing both clinical disease and an asymptomatic carrier condition (*25*). The survival of *Salmonella* in these asymptomatic carriers may lead to significant problems by unintentional transmission of *Salmonella* to both naive animals and humans.

Many *Salmonella* serovars can reside in swine generating opportunities for distribution to humans by contaminated pigs and pork products (*22*, *23*). *Salmonella* populations infecting swine farms are continually dynamic, but some of the most commonly isolated non-clinical serovars include *Salmonella enterica* serovars Bredeney, Derby, Heidelberg, Typhimurium, and Worthington (*2*, *5*, *9*, *17*, *20*).

In field studies, *Salmonella* transmission from infected pigs to naive pigs has been shown to occur during the short period of time pigs are transported to slaughter and while in lairage (*10*, *12-15*, *17, 18*). Pen mates of selected animals whose *Salmonella* status was determined at their farms of origin by both antemortem and postmortem techniques were found to be colonized with a more diverse population of *Salmonella* serovars after slaughter as compared to on the farm indicating *Salmonella* transmission either at the farm, during transport, or while being held in lairage (*14*, *15*).

Acute infection in swine was first experimentally demonstrated when virulent *Salmonella* serovar Typhimurium strain χ4232 was isolated from intestinal and non-intestinal

tissues three hours after intranasal challenge of esophagostomized pigs (*11*). This indicated that *Salmonella* are capable of rapidly infecting internal organs and the intestinal tract through means other than enterocyte and intestinal M cell invasion within 3 hours after inoculation.

This study was conducted to experimentally evaluate the ability of frequently isolated wild-type lymph node isolates, wild-type fecal isolates, and other well-characterized strains of *Salmonella* to acutely infect swine.

Materials and Methods

Isolation Rooms. Rooms were cleaned and disinfected with Deterg™ (Airkem Professional Products, USA) as instructed by the manufacturer. Four days immediately prior to pig arrival, BBL "cultureswabs" containing Stuart's medium (Fisher Healthcare, USA) were used to take randomly located and sized environmental samples of the nursery decks and from various locations in the isolation rooms. Swabs were processed by directly plating them onto 5% Columbia Sheep blood agar plates (Remel, USA) and screened for *Salmonella* by placing them into 9 ml of buffered peptone water (BPW) (Becton Dickinson, Difco, USA), and incubated at 37°C for 18-24 hours. Samples were vortexed, and selectively enriched by transferring 0.1 ml BPW to 9.9 ml of Rappaport-Vassiliadis R10 broth (RV) (Becton Dickinson, Difco) which was incubated at 42°C for 24 hours. Following incubation the RV broth was vortexed, and plated for isolation onto xylose lysine deoxycholate (XLD) agar (Becton Dickinson, Difco).

Animals. Crossbred pigs, 10 to 14 days old, were obtained from 3 local farms. Pigs were transported to Iowa State University where they were randomly assigned to groups of 4. The pigs were placed on nursery decks in isolation rooms, given water and irradiated feed ad

lib, and acclimated for 5 to 10 days. During acclimation, fecal and serum samples were obtained to verify that the pigs were free of *Salmonella* by culture and Danish mix-ELISA (*19*). Seven experiments were conducted which included 1 or 2 uninoculated control pigs and 1-3 principal groups of 4 pigs each. All animal research was conducted under compliance with Iowa State University and federal government guidelines.

Bacterial Strains. *Salmonella* wildtype isolates were isolated from lymph nodes or feces respectively. Lymph node isolates were isolated from ileocecal lymph nodes of *Salmonella*-positive pigs from a Midwest slaughter plant (*7*). Fecal isolates had been previously isolated from pooled pen fecal samples from swine farms throughout the United States (*8*) (Table 1). All wildtype isolates were serotyped at the National Veterinary Services Laboratories (Ames, Iowa). Vaccine strain *Salmonella* serovar Choleraesuis strain SC-54 previously attenuated by neutrophil passage, and its virulent parent strain SC-38 were obtained from Boehringer Ingelheim Vetmedica (Iowa, USA) (*21*). Vaccine strain *Salmonella* serovar Choleraesuis strain Argus was obtained from InterVet (Wisconsin, USA). *Salmonella* serovar Choleraesuis strain Argus is a Δcya/ Δcrp mutant (*3*). Strain *Salmonella* serovar Typhimurium χ4232 was derived from strain 798 (*25*) which was genetically modified to be naladixic acid resistant by RA Curtiss, III, and obtained from the United States Department of Agriculture, National Animal Disease Center. The *Salmonella* serovar Typhimurium variant Copenhagen isolates were phage typed at the National Veterinary Services Laboratories (Ames, Iowa).

Challenge/Necropsy. *Salmonella* cultures were grown overnight in Luria-Bertani Miller (LB) broth (Becton Dickinson, Difco). One-half ml of the static overnight culture was inoculated into 50 ml of fresh LB broth. The organisms were grown to late log phase, and

centrifuged at 5,000 RPM for 15 minutes at 5°C. The cell pellet was resuspended in phosphate buffered saline to a predetermined optical density to yield approximately 5 X 10^9 cells per ml. An 18-gauge 1.88-inch Angiocath™ catheter (Becton Dickinson, Difco) was utilized to inoculate 0.5 ml of the *Salmonella* suspension into each naris of the experimental animals. Three hours post inoculation the animals were euthanized with Beuthanasia™ (Schering-Plough Animal Health, USA). Approximately 5 grams of tonsil, mandibular lymph node, thymus, lung, liver, spleen, colon contents, ileocecal lymph node, and 20 grams of cecum contents were aseptically collected for isolation of *Salmonella*. Twenty milliliters of blood were obtained from the axillary artery of each animal and placed into EDTA tubes for isolation of *Salmonella*.

***Salmonella* Culture.** Tissue samples were bluntly macerated with a rubber mallet. Following maceration all samples were pre-enriched by diluting them 1:9 in BPW, and incubated at 37°C for 18-24 hours. Samples were vortexed, and selectively enriched by transferring 0.1 ml BPW to 9.9 ml of RV broth and incubated at 42°C for 24 hours. Following incubation the RV broth was vortexed, and plated for isolation onto XLD agar (*6*).

***Salmonella* Isolation.** Colonies typical of *Salmonella* were taken from the XLD agar, and inoculated into triple sugar iron agar, lysine iron agar, phenylalanine agar, sulfide indole motility agar, and onto tryptic soy agar and incubated for 24 hours at 37° C. The isolates were not re-serotyped, but were presumptively confirmed as *Salmonella* and serogrouped with Bacto™ monovalent specific "O" antisera (Becton Dickinson, Difco). Data for each sample was recorded as to the presence or absence of *Salmonella* colonies.

Results

All swabs of the isolation rooms, including nursery decks, suggested the rooms to be free of *Salmonella* prior to the study's initiation. All fecal swabs were found to be free of *Salmonella*, and all serum antibody levels were found to be within normal limits as determined by the Danish mix-ELISA.

In studies reported here, of the *Salmonella* isolates tested *Salmonella* serovar Typhimurium χ4232 and *Salmonella* serovar Heidelberg displayed some of the highest percentages of acute infection (Table 2). All *Salmonella* serovars, except *Salmonella* serovar Choleraesuis strain SC-54, were isolated from both alimentary and non-alimentary tissues. *Salmonella* serovar Choleraesuis strain SC-54 was not isolated from alimentary tissues of any of the 4 pigs challenged with this strain, and disseminated to only 4 versus 18 of 28 non-alimentary tissues when compared to its virulent parent *Salmonella* serovar Choleraesuis strain SC-38.

Of the 9 control pigs, 3 animals were positive for *Salmonella* at necropsy. Of these positive animals 0 of 33 alimentary tissues contained *Salmonella* while 4 of 77 non-alimentary tissues (one thymus sample, one spleen sample, one ileocecal lymph node sample, and one blood sample) were positive for the organism. Each of the 4 contaminate isolates was determined to be of a different serogroup than the serogroup inoculated.

Discussion

All wild-type lymph node and fecally derived serovars of *Salmonella enterica* infected both alimentary and non-alimentary tissues, though to different degrees, within 3 hours after being intranasally inoculated into experimental pigs. Known virulent isolates of *Salmonella* serovar Typhimurium and *Salmonella* serovar Choleraesuis also acutely infected

both alimentary and non-alimentary tissues as determined by this model. The degree of re-isolation by avirulent vaccine strains was qualitatively reduced and thus readily differentiated from wild-type and known virulent isolates. *Salmonella* was consistently isolated from the tonsils, excluding the vaccine strains, which supports the findings by Wood *et al.* that the tonsils are a frequent site of *Salmonella* colonization (*25*). The frequent colonization of the tonsil, which serves as an important antigen sampling site, may indicate the tonsils to be a possible point of entry for the dissemination of *Salmonella*. The ability to isolate *Salmonella* from blood 3 hours following challenge may indicate a hematogenous mechanism of acute infection, and thus help differentiate *Salmonella* carriers from acutely infected animals. It can be assumed that wild-type field isolates of a variety of serovars, when encountered at high doses, can be transmitted from pig to pig in the short period of time that animals are either transported or held in lairage prior to slaughter. Lower inoculum doses, which are more commonly found in lairage environments, may not acutely infect pigs to similar degrees compared to higher challenge doses.

It has been suggested that preventing co-mingling of pigs from herds with high prevalences of *Salmonella* with pigs with low prevalences during transport and slaughter may reduce transmission of *Salmonella* caused by acute infection (*1*). This indicates that reduction of acute infection during transport and lairage is possible, but due to current producer and consumer limitations in the United States these types of interventions are unlikely to be implemented.

Salmonella serovar Typhimurium and *Salmonella* serovar Heidelberg were found to be amongst the most commonly isolated serovars in epidemiologic studies (*5*, *17*, *20*). *Salmonella* serovar Typhimurium χ4232 and *Salmonella* serovar Heidelberg displayed high

percentages of acute infection to both alimentary and non-alimentary tissues. The high prevalence of these serovars in swine and the increased ability of these serovars to acutely infect tissue may explain why these isolates are reported to have the highest incidence of swine associated human salmonellosis cases in the United States (*23*). The ability of *Salmonella* to acutely infect tissues classified as offal, which are commonly used in the production of pork sausages, may indicate why sausages are frequently found to harbor *Salmonella (24)*. Although *Salmonella* serovar 6, 7 non-motile displayed the highest percentages of acute infection this serovar is rarely isolated from swine (*5*, *17*, *20*).

Three uninoculated control animals (4 of 77 non-alimentary samples) were found to be *Salmonella* positive at necropsy. Since these isolates were of a different serogroup than was inoculated into the cohort group, it was assumed these isolates originated from the source farm, or from the isolation rooms. Culture swabs of the isolation rooms may have given false negative results due the lack of sensitivity when culturing with swabs. Successful identification of pigs with *Salmonella* from the source farm may not have occurred as pigs to harbor and fecally shed non-clinical *Salmonella* in inconsistent patterns (*25*). These shedding patterns may have allowed *Salmonella* positive animals to go undetected prior to the study's initiation.

Variation in *Salmonella* serovar virulence may play an important role in the ability and speed at which *Salmonella* serovars can disseminate throughout the pig. Significant variation between dissemination to alimentary and non-alimentary tissues was not seen amongst wild-type isolates, but notable differences were seen when comparing virulent strains with avirulent *Salmonella* serovar Choleraesuis vaccine strains. Reduction in the number of tissues infected by virulent *Salmonella* serovar Choleraesuis SC-38 compared to

its avirulent progeny, *Salmonella* serovar Choleraesuis SC-54, indicates that virulence attenuation may greatly effect acute infection. Since *S.* serovar Choleraesuis strain SC-54 and strain Argus were altered by attenuation and mutation respectively, culture data alone may be misleading due the possibility that these strains may have disseminated but remained incapable of replication in non-alimentary tissues. Specifically, *Salmonella* Δcya/ Δcrp mutants may exhibit altered metabolic functions due to alteration of adenylate cyclase and cAMP receptor protein genes. Altered cAMP levels may decrease the ability of these mutants to survive and replicate in nutritionally diverse areas such as those seen in non-alimentary tissues (*4*). Though not conducted here, utilization of a realtime quantitative polymerase chain reaction could be utilized to determine if *Salmonella* specific DNA in these culture negative tissues was present (*16*).

Previously, it has been shown that *Salmonella* serovar Typhimurium can disseminate to internal tissues in as quickly as 2 hours post infection (*12*). Our study has indicated that many other *Salmonella* serovars also posses the ability to rapidly disseminate and infect tissues within three hours after inoculation indicating their importance in acute infection of swine immediately prior to slaughter.

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Table 1. Isolates of *Salmonella enterica* inoculated into experimental pigs

Organism	**ISU isolate #**	**Isolate Source**
S. 4,12:i-monophasic	HL10371	Lymph node
S. 6,7 non-motile	HL10225	Lymph node
S. Agona	HL10170	Feces
S. Brandenburg	HL10177	Feces
S. Bredeney	HL10024	Feces
S. Choleraesuis Argus	HL10806	InterVet (Wilson)
S. Choleraesuis SC-38	HL10805	Boehringer Ingelheim Vetmedica (Roof)
S. Choleraesuis SC-54	HL10803	Boehringer Ingelheim Vetmedica (Roof)
S. Derby	HL10342	Lymph node
S. Heidelberg	HL10374	Lymph node
S. Infantis	HL10343	Lymph node
S. Muenchen	HL10013	Feces
S. Thompson	HL10189	Feces
S. Typhimurium	HL10426	Lymph node
S. Typhimurium var. Copenhagen DT104	HL10222	Feces
S. Typhimurium χ4232	HL10969	National Animal Disease Center (Hurd)
S. untypeable	HL10416	Lymph node
S. Worthington	HL10356	Lymph node

Table 2. Number of tissues positive for *Salmonella* serovars

	Lymph Node Isolates (n=4)*								Fecal Isolates (n=4)						Vaccine Strains (n=4)		Virulent Strains (n=4)		Controls (n=11)
	Salmonella 6,7 non-motile	S. Heidelberg	S. Worthington	S. *4,12*:i-monophasic	S. Infantis	S. Derby	*Salmonella* untypeable	S. Typhimurium var. Cop	S. Typhimurium var. Cop DT104	S. Thompson	S. Bredeney	S. Agona	S. Muenchen	S. Brandenburg	S. Choleraesuis Argus	S. Choleraesuis SC-54	S. Typhimurium x4232	S. Choleraesuis SC-38	Uninoculated
Alimentary Tissues																			
Tonsil	4	4	4	3	4	4	4	4	4	4	4	4	4	4	0	0	4	4	0
Colon Contents	3	2	3	4	2	3	3	2	2	3	2	3	1	2	1	0	4	1	0
Cecum Contents	4	4	4	4	4	4	4	3	4	4	3	4	3	2	2	0	4	3	0
% Positive	**92**	**83**	**92**	**92**	**83**	**92**	**92**	**75**	**83**	**92**	**75**	**92**	**67**	**67**	**25**	**0**	**100**	**67**	**0**
Non-Alimentary Tissues																			
Mandibular Lymph node	4	2	4	4	4	1	1	4	4	2	2	2	2	3	1	0	3	3	0
Thymus	4	4	2	0	2	2	0	1	4	3	3	2	4	2	0	0	3	4	1
Lung	4	4	2	1	2	2	1	2	2	2	4	2	2	0	1	1	4	4	0
Liver	4	3	2	3	1	2	3	1	3	3	0	1	1	1	1	2	3	3	0
Spleen	4	4	2	2	1	2	1	1	4	1	1	1	0	2	0	1	4	2	1
Ileocecal Lymph node	4	4	3	2	1	2	3	0	4	2	3	2	2	2	2	0	3	2	1
Blood	4	2	0	1	2	0	1	0	0	4	1	2	1	1	0	0	2	0	1
% Positive	**100**	**82**	**54**	**46**	**46**	**39**	**36**	**32**	**75**	**61**	**50**	**43**	**43**	**39**	**18**	**14**	**79**	**64**	**5**
% Positive (All Tissues)	**98**	**83**	**68**	**60**	**56**	**55**	**53**	**45**	**78**	**70**	**58**	**58**	**53**	**48**	**20**	**10**	**85**	**65**	**4**

* Number in parenthesis indicates the number of pigs inoculated and tissues sampled per serovar or uninoculated control

CHAPTER 3: ACUTE INFECTION OF SWINE BY VARIOUS MUTANT *SALMONELLA*

A paper prepared for publication in *The Journal of Food Protection*

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Introduction

Acute *Salmonella* infection has been extensively studied in transportation and lairage situations but little has been done to determine the mechanisms by which *Salmonella* acutely infect pigs. *Salmonella* are most frequently transmitted by the fecal-oral route, though direct contact and airborne transmission have been described (14). Following ingestion in chronic infection models, *Salmonella* are capable of traveling aborally down the alimentary tract infecting tissues they encounter. Particles similar in size to bacteria and liquid markers are capable of reaching the distal intestinal tract within 2 hours of oral uptake indicating this to be a possible mechanism of alimentary tract acute infection (1). Following infection of the distal small intestine, *Salmonella* can invade the gut wall using multiple mechanisms and cause systemic infection (10-12, 17-19). Fedorka-Cray *et al.* experimentally demonstrated acute *Salmonella* infection in pigs when virulent *S.* Typhimurium strain χ4232were isolated from alimentary and non-alimentary tissues 3 hours after intranasal challenge in esophagostomized pigs (9). This suggested that *Salmonella* are capable of a novel mode of invasion that does not require invasion of the gastrointestinal tract to cause systemic dissemination, as previously thought.

Many *Salmonella* strains have been manipulated to express foreign genes (3) or inactivate certain *Salmonella* virulence genes (2, 5, 16). These altered mutant and attenuated strains have be used to understand the gene functions in bacterial pathogenicity and

biochemical operations. The ability or inability of certain *Salmonella* strains to acutely infect swine may lead to a better understanding of the mechanisms behind acute infection, and hopefully lead to the development effective interventions.

The following study was conducted to evaluate the ability of rare swine *Salmonella* isolates, mutant *Salmonella* strains, and commercially available avirulent *Salmonella* vaccine strains to acutely infect both alimentary and non-alimentary tissues in order to help identify a possible mechanism of acute *Salmonella* infection.

Materials and Methods

Isolation rooms. Rooms were cleaned and disinfected with Deterg™ (Airkem Professional Products, USA) as instructed by the manufacturer. Four days immediately prior to pig arrival, BBL "cultureswabs" containing Stuart's medium (Fisher Healthcare, USA) were used to take randomly sized environmental samples from random locations of the nursery decks and isolation rooms. Swabs were processed by directly plating them onto 5 % Columbia Sheep blood agar plates (Remel, USA), and then screened for *Salmonella* by placing them into 9 ml of buffered peptone water (BPW) (Becton Dickinson, Difco, USA) and incubating them at 37° C for 18 to 24 hours. Samples were vortexed and selectively enriched by transferring 0.1 ml BPW to 9.9 ml of Rappaport-Vassiliadis R10 broth (RV) (Becton Dickinson, Difco), which was incubated at 42°C for 24 hours. Following incubation the RV broth was vortexed and plated for isolation onto xylose lysine deoxycholate (XLD) agar (Becton Dickinson, Difco).

Animals. Crossbred pigs, 10 to 14 days old, were obtained from 3 local farms. The pigs were placed on nursery decks in isolation rooms, given water and irradiated feed ad lib, and acclimated for 5 to 10 days. During acclimation, fecal and serum samples were obtained

to verify by culture and Danish mix-ELISA (13) that the pigs were free of *Salmonella* and significant levels of *Salmonella* antibodies. Seven experiments, which included 1 or 2 uninoculated control pigs and 1-3 principal groups of 4 pigs each, were conducted. All animal research was conducted in compliance with Iowa State University and federal government guidelines.

Virulent *Salmonella* strains. Wild-type isolate *Salmonella* 6,7 non-motile was isolated from an ileocecal lymph node of a *Salmonella*-positive pig from a Midwest slaughter plant (7) and serotyped at the National Veterinary Services Laboratories (Ames, Iowa). *Salmonella* Choleraesuis strain SC-38 was obtained from Boehringer Ingelheim Vetmedica (Iowa, USA). *Salmonella* serovar Typhimurium strain χ4232 was derived from strain 798 (18), which was genetically modified to be naladixic acid resistant by R.A. Curtiss, III and obtained from the United States Department of Agriculture National Animal Disease Center. *Salmonella* serovar Typhimurium strain χ4232pp was pig passaged by recovering the isolate from the ileocecal lymph node of a pig inoculated with 5 x 10^9 organisms of *Salmonella* serovar Typhimurium strain χ4232. *Salmonella* serovar Typhimurium strain χ4347 is a virulent tetracycline resistant strain received from R.A. Curtis, III (University of Washington, St. Louis, MO, USA).

Avirulent and mutant *Salmonella* strains. Vaccine strain *Salmonella* Choleraesuis strain SC-54, previously attenuated by neutrophil passage of its parental strain SC-38, was obtained from Boehringer Ingelheim Vetmedica (15). Vaccine strain *Salmonella* serovar Choleraesuis strain Argus was obtained from InterVet (Wisconsin, USA). *Salmonella* Choleraesuis strain Argus is a Δcya/ Δcrp mutant (4). *Salmonella* Typhimurium strain χ4233 is a Δcya/ Δcrp mutant and was obtained from RA Curtis, III. *Salmonella* Typhimurium

strain SL 1344 is a SifA deletion mutant and was obtained from BB Finlay (University of British Columbia, Vancouver, BC, Canada) (5). *Salmonella* serovar Typhimurium strain SL 7541 is an aroA(serC)::Tn10 insertion mutant and was obtained from BAD Stocker (Stanford University, Stanford, CA, USA) (16).

Challenge/necropsy. *Salmonella* cultures were grown overnight in Luria-Bertani Miller (LB) broth (Becton Dickinson, Difco). One-half ml of the static overnight culture was inoculated into 50 ml of fresh LB broth. The organisms were grown to late log phase and centrifuged at 5,000 RPM for 15 minutes at 5° C. The cell pellet was resuspended in phosphate buffered saline to a predetermined optical density to yield approximately 5 x 10^9 cells per ml. An 18-gauge 1.88-inch Angiocath™ catheter (Becton Dickinson, Difco) was utilized to inoculate 0.5 ml of the *Salmonella* suspension into each naris of the experimental animals. Three hours post inoculation the animals were euthanized with Beuthanasia™ (Schering-Plough Animal Health, USA). Approximately 5 g of tonsil, mandibular lymph node, thymus, lung, liver, spleen, colon contents, ileocecal lymph node, and 20 g of cecum contents were aseptically collected for isolation of *Salmonella*. Twenty ml of blood were obtained from the axillary artery of each animal and placed into EDTA tubes until processed.

***Salmonella* culture.** Tissue samples were bluntly macerated with a rubber mallet. Following maceration all samples were pre-enriched by diluting them 1:9 in BPW and incubated at 37° C for 18 to 24 hours. Samples were vortexed, selectively enriched by transferring 0.1 ml BPW to 9.9 ml of RV broth, and incubated at 42° C for 24 hours. Following incubation the RV broth was vortexed and the cultures were plated for isolation onto XLD agar (6).

***Salmonella* isolation**. Colonies typical of *Salmonella* were taken from the XLD agar; inoculated into triple sugar iron agar, lysine iron agar, phenylalanine agar, sulfide indole motility agar, and onto tryptic soy agar; and incubated for 24 hours at 37° C. The isolates were not re-serotyped, but were presumptively confirmed as *Salmonella* and serogrouped with Bacto™ monovalent specific "O" antisera (Becton Dickinson, Difco). Data for each sample were recorded as to the presence or absence of *Salmonella* colonies.

Results

Virulent, avirulent, and mutant strains of *Salmonella* were capable of acutely infecting non-alimentary tissues, and all strains but SC-54 were capable of infecting alimentary tissues (Table 1). Both avirulent/mutant strains and virulent strains were capable of causing acute infection, but the frequency of *Salmonella* isolation varied depending on the individual strain.

Pigs inoculated with *Salmonella* Typhimurium strain SL 1344 exhibited the greatest frequency of acute infection to the alimentary tissues of all avirulent/mutant strains studied (75% alimentary dissemination), while *Salmonella* Choleraesuis strain SC-54 displayed the least amount of acute infection to alimentary tissues (0% alimentary dissemination) (Table 1). *Salmonella* Typhimurium strain SL 1344 exhibited the greatest frequency of acute infection to the non-alimentary tissues of all avirulent/mutant strains studied (75% non-alimentary dissemination) while *Salmonella* Typhimurium strain SL 7541 displayed the least amount of acute infection to non-alimentary tissues (7% non-alimentary dissemination).

Salmonella Typhimurium χ4232pp exhibited the greatest frequency of acute infection to the alimentary tissues of all virulent strains studied (100% alimentary dissemination) while *Salmonella* Choleraesuis strain SC-38 exhibited the least amount of acute infection to the

alimentary tissues (67% alimentary dissemination). *Salmonella* 6,7 non-motile exhibited the greatest frequency of acute infection to the non-alimentary tissues of all virulent strains studied (100% non-alimentary dissemination), while *Salmonella* Typhimurium strain χ4232 exhibited the least amount of acute infection to the non-alimentary tissues (17% non-alimentary dissemination).

Salmonella Δcya/crp mutants (*Salmonella* Typhimurium strain χ4233 and *Salmonella* Choleraesuis strain Argus) and *Salmonella* vaccine strain SC-54 displayed the least amount of acute infection. Strains χ4233, Argus, and SC-54 disseminated to all tissues at rates of 15%, 20%, and 10%, respectively.

Of the 7 control pigs, 2 animals were positive for *Salmonella* at necropsy. Of these positive animals, 0 of 21 alimentary tissues contained *Salmonella* while 3 of 49 non-alimentary tissues (one spleen sample, one ileocecal lymph node sample, and one blood sample) were positive for the organism. Each of the 4 contaminate isolates was determined to be of a different serogroup than any of the serogroups inoculated into the principal pigs.

Discussion

Virulent and avirulent *Salmonella* were capable of acutely infecting both alimentary and non-alimentary tissues within 3 hours after intranasal inoculation, with the exception of *S.* Typhimurium strain SC-54, which did not acutely infect alimentary tissues. Wide degrees of variation in *Salmonella* isolation were observed among all the *Salmonella* serovars tested.

Virulent *S.* Choleraesuis strain SC-38 disseminated more frequently to both alimentary and non-alimentary tissues compared to avirulent *S.* Choleraesuis vaccine strains SC-54 and Argus. Both avirulent *S.* Choleraesuis strains disseminated similarly to the non-

alimentary tissues. *S.* Choleraesuis strain SC-54 was not isolated from any of the alimentary tissues, while *S.* Choleraesuis strain Argus was isolated from 25% of the alimentary tissues.

S. Choleraesuis strain SC-38 was attenuated by serial neutrophil passage producing *S.* Choleraesuis strain SC-54, which was cured of a 50 kb plasmid (15). The 50 kb plasmid has been associated with increased resistance to hydrogen peroxide, increased resistance to phagocytic killing by neutrophils, and a decreased ability to invade Vero cell monolayers. The decreased isolation of SC-54 from non-alimentary tissues may indicate the importance of isolate invasion on acute *Salmonella* infection. The lack of SC-54 isolation from alimentary tissues cannot be explained when comparing it to its virulent parental strain SC-38.

Erdman *et al.* have shown that pigs inoculated with avirulent vaccines strains, Argus and SC-54, were incapable of producing large amounts of neutralizing antibodies and were unable to reduce acute *Salmonella* infection in pigs (8). The decreased ability of these vaccine strains to induce acute *Salmonella* infection, as presented in these studies, may indicate that the vaccine strains were not invasive enough to precipitate the needed immune response to reduce acute *Salmonella* infection.

Strains *S.* Typhimurium strain χ4233 and *S.* Choleraesuis strain Argus exhibited reduced acute infection when compared to their parental strains χ4232 and SC-38, respectively. Strains χ4233 and Argus are Δcya/crp mutants capable of attaching, invading, and surviving in host tissue at levels comparable to their virulent counterparts, but have been shown to have a decreased ability to colonize both alimentary and non-alimentary tissues. The cya/crp genes are important for the regulation of adenylate cylcase and the cAMP receptor proteins, which ultimately aid in glucose utilization, amino acid metabolism, synthesis of flagella and pili, and synthesis of certain *Salmonella* outer surface proteins.

Reduced acute infection of Δcya/crp mutants from both alimentary and non-alimentary tissues may be the result of either decreased growth due to a lack of energy producing metabolites, or a decrease in the production of flagella, pili, or outer membrane proteins that are important in phagocytic uptake.

All virulent *Salmonella* strains acutely infected alimentary tissues to a similar degree with *Salmonella* 6,7 non-motile disseminating to the most tissues of any strain tested (11 of 12 alimentary tissues and 28 of 28 non-alimentary tissues). Strain χ4232 disseminated to non-alimentary tissues to a lesser degree when compared to other virulent *S.* Typhimurium strains. Pig passaged *S.* Typhimurium χ4232pp disseminated similarly to alimentary tissues and disseminated more frequently to non-alimentary tissues compared to its non-pig passaged counterpart χ4232. *Salmonella* passage through pigs, which likely occurs during holding immediately prior to slaughter, may increase the ability of isolates to acutely infect animals housed in the same holding pens. This increase in acute infection virulence may indicate that somatic o-antigens, upregulation of *Salmonella* outer membrane proteins, or increased host-killing resistance after pig passage may play an influential role in acute *Salmonella* infection.

The greatest variation was observed betweem *S.* Typhimurium avirulent and mutant strains. Strains SL1344 and SL7541 had acute infection rates similar to those of the virulent strains tested. *S.* Typhimurium strain χ4233 was the only avirulent mutant to exhibit reduced acute infection. Strain SL1344 is a ΔsifA mutant which has the inability to form *Salmonella* induced filaments which aid in macrophage colonization. The ΔsifA mutants were not capable of surviving and replicating at levels comparable to their parental strain in murine macrophages (2). Since strain SL1344 is capable of acutely infecting pigs at levels

comparable to virulent strains it is not likely that survival and replication in macrophages plays an important role in acute *Salmonella* infection. Strain SL7541 is an aroA(serC)::Tn10 insertion mutant. aro*A* mutants have undergone attenuation that greatly increase *Salmonella* lethal doses, but do not negatively affect immunogenic properties (16). These mutants have loss ser*C* gene function, which requires these strains to be provided with serine, pyridoxine, and aromatic amino acids. The ability to isolate SL7541 from non-alimentary tissues may be considered surprising due to the decreased availability of aromatic amino acids in non-alimentary tissues, but is likely explained due to the short time period between *Salmonella* infection and necropsy allowing the organism to survive without replicating in the host tissues. The lack of reduction of acute infection by SL7541 suggests that virulence factors associated with increased mortality, as expressed by LD_{50} calculations, do not seem to play an important role in acute infection virulence.

It has been suggested that avirulent *Salmonella* strains that are capable of disseminating to a wide range of tissues may serve as ideal vaccine candidates by allowing systemic immune responses without inducing disease processes (16). When analyzing acute infection of wild-type *S.* Choleraesuis strains and *S.* Choleraesuis vaccine strains, which provide protection against systemic salmonellosis in swine, it can be noted that the vaccines do not acutely infect a wide variety of tissues. This lack of vaccine dissemination contrarily suggests that effective *Salmonella* vaccines do not require systemic infection in order to produce protective immunity.

Two uninoculated control animals (3 of 49 non-alimentary samples) were found to be positive for *Salmonella* at necropsy. Since these isolates were of a different serogroup than what was inoculated into the cohort groups, it was assumed these isolates originated from the

source farm or the isolation rooms. Culture swabs of the isolation rooms may have given false negative results due to the lack of sensitivity when culturing with swabs. Successful identification of pigs with *Salmonella* from the source farm may not have been accomplished because of the ability of pigs to harbor and fecally shed non-clinical *Salmonella* in inconsistent patterns (18). These shedding patterns may have allowed *Salmonella* positive animals to go undetected prior to the study's initiation.

This study suggests that acute *Salmonella* infection varies based on deletion or inactivation of certain genes. Most notably, reduction in acute *Salmonella* infection was seen in avirulent deletion mutants in which the cya/crp genes had been inactivated or in those strains attenuated by neutrophil passage. Non-motile *Salmonella* disseminated to the greatest number of tissues of any of the *Salmonella* examined. Continual passage of virulent strains through the pig may enhance or up regulate important factors associated with acute *Salmonella* infection, which may ultimately increase the ability of certain strains to acutely infect pigs immediately prior to slaughter.

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Table 1. Acute *Salmonella* infection by various mutant *Salmonella*

	Mutant Strains			Avirulent Strains		Virulent strains					Controls
	S. Typhimurium			S. Choleraesuis		S. Typhimurium			S. Choleraesuis	Other	
n=4	SL1344[A]	SL 7541[B]	x4233[C]	Argus[D**]	SC-54[E**]	x4232pp[F]	x4347[G]	x4232[H**] (n=6)	SC-38[I**]	*Salmonella* 6,7 non-motile[J**]	Uninoculated (n=7)
Alimentary Tissues											
Tonsil	4	4	1	0	0	4	4	6	4	4	0
Colon Contents	1	1	2	1	0	4	2	4	1	3	0
Cecum Contents	4	1	1	2	0	4	4	6	3	4	0
% Positive	75	**50**	**33**	**25**	**0**	**100**	**83**	**89**	**67**	**92**	**0**
Non-Alimentary Tissues											
Mandibular Lymph node	4	3	1	1	0	3	4	3	3	4	0
Thymus	4	3	0	0	0	3	4	ж	4	4	0
Lung	2	4	0	1	1	4	3	0	4	4	0
Liver	3	1	1	1	2	3	2	2	3	4	0
Spleen	4	4	0	0	1	4	3	1	2	4	1
Ileocecal Lymph node	4	4	0	2	0	3	3	0	2	4	1
Blood	0	0	0	0	0	2	0	0	0	4	1
% Positive	**75**	**68**	**7**	**18**	**14**	**79**	**68**	**17**	**64**	**100**	**6**
% Positive (All Tissues)	**75**	**63**	**15**	**20**	**10**	**85**	**73**	**41**	**65**	**98**	**4**

Note-- The number in parenthesis indicates the number of pigs inoculated and tissues sampled per serovar or uninoculated control

** This data was previously published in chapter 2.

ж Not done

A= Δ SifA

B= aroA(serC)::Tn10

C= Δ cya/crp

D= Δ cya/crp

E= neutrophil passaged attenuation

F= virulent; pig passaged x4232

G= virulent; tetracycline resistant

H= virulent; naladixic acid resistant

I= virulent

J= rare swine isolate

CHAPTER 4: DOSE DETERMINATION FOR ACUTE *SALMONELLA* INFECTION IN PIGS

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Introduction

Pigs frequently harbor *Salmonella* subclinically allowing the organism to be transmitted amongst pigs prior to slaughter (4,6,7,10). Transmission of *Salmonella* from pigs with subclinical infections to naïve pigs during transportation and lairage has been proposed to be a major source of *Salmonella* introduction into the food chain (5,10). Events immediately prior to slaughter have been shown to correlate with an increased rate of *Salmonella* isolation from pig carcasses (5,6) and from pork products (2,8,12).

Previous work in our laboratory has shown numerous *Salmonella* serovars are capable of acutely infecting both alimentary and non-alimentary tract tissues within 3 hours after intranasal inoculation (9). Acute *Salmonella* infection has been shown to occur in market weight pigs after exposed to a contaminated environment at doses comparable to those reported in lairage ($<1 \times 10^6$) (4).

The objectives of this study were to determine the minimum dose required to induce acute *Salmonella* infection in pigs intranasally inoculated with *Salmonella* (trials 1 and 2), and evaluate acute *Salmonella* infection in pigs exposed to a contaminated environment containing various levels of *Salmonella* (trials 3 and 4).

Materials and Methods

Isolation rooms. Prior to pig arrival, drag swab samples of the rooms were pre-enriched in buffered peptone water (Becton Dickinson, Difco), selectively enriched in

Rappaport Vassiliadis broth (Becton Dickinson, Difco), selectively plated on xylose lysine deoxycholate agar (XLD) (Becton Dickinson, Difco), and then *Salmonella* suspect colonies were picked to differential biochemical media as previously described. (9)

Animals. Crossbred pigs, 10 to 14 days old, were randomly assigned to 1 of 3 principal groups (5 animals per group) or to a negative control group. The pigs were acclimatized for 7 to 14 days in isolation rooms, and given water and irradiated feed (Harlan Teklad, WI) ad lib. During acclimatization, rectal swabs and pooled pen fecal samples (3) were obtained to verify the pigs to be free of detectable *Salmonella.*

Salmonella. The challenge strain, *S. enterica* subspecies *enterica* serovar Typhimurium strain HL 10969 was derived from a naladixic acid resistant strain χ4232, which was genetically modified to produce green florescent protein (GFP) as previously described (1). The isolate was selected for increased acute infection virulence determinates by inoculating the organism into pigs, and re-isolating it from the ileocecal lymph node 3 hours later.

Strain HL 10969 was grown to late log phase in Lauria-Bertani Miller broth (Becton Dickinson, Difco), and centrifuged at 5,000 RPM for 15 minutes at 5° C. The cell pellet was washed in phosphate buffered saline (PBS) and centrifuged two additional times. Following the 3rd centrifugation step the cell pellet was resuspended in PBS containing 20% glycerol, and frozen at -80° C.

Challenge/Necropsy. *Intranasal challenge, trials 1 and 2.* Strain HL 10969 was removed from the freezer and serially diluted to 4.5 X 10^5, 4.2 X 10^3, and 4.8 X 10^1 (trial 1), or 2.8 X 10^7, 2.8 X 10^5, and 2.8 X 10^3 (trial 2) organisms per ml as determined by viable plate counts. Animals were intranasally inoculated as previously described (14).

Contaminated environment challenge, trials 3 and 4. During acclimatization, feces were collected and stored at 4° C until the day of challenge. Five days prior to challenge the pooled feces were verified to be free of *Salmonella* by pre-enrichment, selective enrichment, and selective plating techniques. Twelve hours before challenge approximately 1 L of physiological saline was added for every 1500 g of feces. The feces was mixed using an electric mixer (Hamilton Beach/ Proctor Silex Inc., NC, USA) on setting 1 for 2 minutes. The feces were then placed into bowls for each *Salmonella* dilution, and the appropriate amount of *Salmonella* was added to obtain 5.2 X 10^4, 5 X 10^2, and 2.5 X 10^1 (trial 3), or 4 X 10^6, 4.1 X 10^4, and 9.1 X 10^2 (trial 4) organisms per gram of feces (PGF) as determined by direct plate counts. Twenty-five grams of the spiked feces were applied to each square foot of the challenge environment.

Animals were euthanized three hours following challenge. Approximately 5 grams of tonsil, mandibular lymph node, thymus, lung, liver, spleen, colon contents, ileocecal lymph node, diaphragmatic muscle, ileum, 20 grams of cecum contents, and 20 ml of blood were collected aseptically for the isolation of *Salmonella.*

***Salmonella* Isolation.** Tissue samples were collected and processed as previously described (9) excluding the selective plating techniques. For the selective plating process samples were plated onto XLD agar containing 50 μg of naladixic acid per ml of agar. Tissues from the negative control animals were processed identically to those of the principal groups, except they were plated for isolation onto XLD agar without naladixic acid. XLD plates were placed under ultraviolet light, and observed for fluorescence typical of the GFP containing challenge strain. Tissues were recorded as to the presence or absence of *Salmonella.*

Calculations. Infectious dose 50 calculations were calculated based on the Reed-Muench equation (http://www.fao.org/DOCREP/005/AC802E/ac802e00.htm).

Results

Salmonella were not isolated from the environmental drag swabs. All rectal swabs and pooled pen fecal samples, taken during acclimatization, were negative for *Salmonella.* All alimentary and non-alimentary tissues from the negative control animals (trials 1, 2, 3, and 4) were culture negative for *Salmonella* at necropsy. The results for intranasal and contaminated environment challenge are presented in Tables 1, 2, and 3.

Discussion

These experiments establish a minimal dose of *Salmonella* needed for acute infection of both alimentary and non-alimentary tissues of swine. An intranasal challenge dose greater than 1 X 10^3 *Salmonellae* is required to infect both alimentary and non-alimentary tissues. The ingestion of *Salmonella* from contaminated environments containing greater than 1 X 10^3 *Salmonellae* per gram of feces induces acute infection of both the alimentary and non-alimentary tissues.

During the movement of swine from farm to slaughter, a variety of conditions such as the number of pigs shedding *Salmonella* into the environment, the length of time in lairage, animal age, breed, concurrent disease status, and stress encountered during transportation and lairage may influence the minimal dose of *Salmonella* required to induce acute infection in pigs. However, these results suggest that reduction of acute *Salmonella* infection may be readily achieved by simple environmental sanitation.

In Denmark, a *Salmonella* control program exists in which farms are categorized into groups of low, medium, or high *Salmonella* (13). This program mandates that pens are

washed between groups, and farms with low prevalence have separate transport, lairage, and slaughter from those with high and medium prevalence. These prophylactic techniques have decreased acute infection as evident by decreased *Salmonella* isolation from pig cecal contents and slaughter carcasses (2,11) possibly by reducing the levels of *Salmonella* below the minimal dose needed to cause acute *Salmonella* infection.

Thus, our results are consistent with the experience of the Danish *Salmonella* control program in that reducing the level of environmental *Salmonella* is adequate for minimizing acute infection by the organism.

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Table 1. Number of positive tissues following intranasal challenges of pigs with various *Salmonella* Typhimurium concentrations.

	Trial 1 (n=5)			Trial 2 (n=5)			Negative controls (n=6)
	1×10^1	1×10^3	1×10^5	1×10^3	1×10^5	1×10^7	
Alimentary Tissues							
Tonsil	0	0	3	1	3	5	0
Ileum	0	0	2	0	3	5	0
Cecum Contents	0	0	2	0	2	5	0
Colon Contents	0	0	0	0	0	5	0
% Positive	**0**	**0**	**35**	**5**	**40**	**100**	0
Non-Alimentary Tissues							0
Mandibular Lymph node	0	0	1	0	1	1	0
Thymus	0	0	0	0	0	1	0
Lung	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0
Spleen	0	0	0	0	0	0	0
Ileocecal Lymph node	0	0	0	0	0	0	0
Muscle	0	0	0	0	0	0	0
Blood	0	0	0	0	0	0	0
% Positive	**0**	**0**	**3**	**0**	**3**	**5**	0
% Positive (All Tissues)	**0**	**0**	**13**	**2**	**15**	**37**	0

Table 2. Number of positive tissues following contaminated environment challenges of pigs with various *Salmonella* Typhimurium concentrations.

	Trial 3 (n=5)			Trial 4 (n=5)			Negative controls (n=4)
	1×10^1	1×10^3	1×10^5	1×10^3	1×10^5	1×10^7	
Alimentary Tissues							
Tonsil	1	0	2	0	2	5	0
Ileum	0	0	1	0	2	4	0
Cecum Contents	0	0	1	0	1	3	0
Colon Contents	0	0	0	0	0	2	0
% Positive	**5**	**0**	**20**	**0**	**25**	**70**	**0**
Non-Alimentary Tissues							
Mandibular Lymph node	0	0	1	0	1	4	0
Thymus	0	0	0	0	0	1	0
Lung	0	0	0	0	0	3	0
Liver	0	0	0	0	1	2	0
Spleen	0	0	0	0	1	3	0
Ileocecal Lymph node	0	0	1	0	1	3	0
Muscle	0	0	0	0	1	2	0
Blood	0	0	0	0	0	2	0
% Positive	**0**	**0**	**5**	**0**	**13**	**50**	**0**
% Positive (All Tissues)	**2**	**0**	**10**	**0**	**17**	**57**	**0**

Table 3. Acute *Salmonella* minimal infectious dose 50 calculations following intranasal challenge.

Alimentary Tissues	Intranasal ID_{50} / mL
Tonsil	1.78×10^5
Ileum	1.48×10^5
Cecum Contents	6.76×10^7
Colon Contents	3.16×10^7

CHAPTER 5: PROBIOTIC USAGE FOR THE REDUCTION OF ACUTE *SALMONELLA* INFECTION IN GERMFREE SWINE

A paper submitted for publication in
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Introduction

Salmonella are capable of acutely infecting alimentary and non-alimentary tissues of pigs during the short time period of transportation and lairage immediately prior to slaughter (17-19, 21) Previous work in our laboratory has indicated that the most prevalent *Salmonella* serovars found in pigs are capable of inducing acute *Salmonella* infection (ASI), and that contaminated environments, such as transportation vehicles and lairage holding pens, which contain over 1 X 10^3 *Salmonella* are sufficient to induce ASI in pigs (24, 25). This rapid infection increases the amount of *Salmonella* found on the carcass and potentially increases the amount of *Salmonella* capable of reaching the consumer (5, 18, 19, 21).

A variety of different intervention methods have been evaluated in their abilities to reduce ASI. The Danish *Salmonella* control program which mandates monthly farm monitoring, categorization of pig herds based on *Salmonella* prevalence, separate transportation and lairage of pigs of varying prevalences, and disinfection of lairage pens between groups of pigs has effectively reduced ASI (32). The cost and intensive nature of the program has hindered its implementation in the U.S., where ASI remains to be problematic. A variety of other interventions have been evaluated but none have consistently reduced ASI (22, 29).

Probiotics are thought to inhibit pathogen infection by binding to pathogens and their associated gastrointestinal receptors, producing microbiotoxic products, competing for

nutrients, and by enhancing the host's immune system (16). Probiotic compositions have provided inconsistent results in reducing *Salmonella* infection in swine. This general lack of efficacy has led some researchers to evaluate probiotics in germfree pigs (6). Germfree pigs have been shown to be very susceptible to *Salmonella* infection (3, 14, 23). A model to evaluate probiotic efficacy in the reduction of ASI has not been previously reported.

Utilization of germfree animals has been shown to be an excellent tool for examining physiological (10, 30), nutritional (26, 33), immunological (7, 8, 11), etiological (20, 28), pathological development of disease (1, 4, 13, 15), and disease intervention strategies (6, 27). Germfree animals have proven to be a good models for these studies due to the lack of the natural microflora, which influence and inhibit gastrointestinal pathogens from establishing. Though numerous studies of *Salmonella* infection in germfree animals have been preformed, germfree animals have never been used to study ASI.

The objectives of this study were to determine if acute *Salmonella* infection occurs in germfree pigs, and to determine if probiotics can reduce acute *Salmonella* infection in pigs.

Materials and Methods

Experimental animals and animal housing. Two pregnant crossbred sows of 111 days gestation were transported to the National Animal Disease Center (NADC) in Ames, Iowa, where at 112 days of gestation hysterotomies were performed producing germfree piglets (31). The piglets were transferred into sterile housing isolators for the duration of the study. Skin and rectal swabs were taken every 4 to 5 days to verify that the isolators were free of bacterial contamination.

Experimental design. Two trials were conducted in which conventional control pigs, germfree pigs, and pigs inoculated with candidate probiotic bacteria were intranasally

challenged 16 days after birth with *Salmonella enterica* subspecies *enterica* serovar Typhimurium isolate HL 10969pp and subsequently necropsied 3 hours following inoculation.

Probiotic preparation. Previous studies, by the authors, confirmed the ability of the probiotics used in this study to colonize both alimentary and non-alimentary tissues of germfree pigs. *Lactobacillus paracasei* subspecies *paracasei* ATCC # PTA-4091 inoculum was prepared, as previously described, to yield approximately 1 X 10^9 organisms per ml (26). An overnight culture of *Bacteroides ovatus* ATCC # PTA-4093 was used to inoculate a 10 ml tube of Sweet E broth (9) which was incubated for 48 hours at 37° C, and titrated on blood agar plates (BAP) (Remel, USA) under anaerobic conditions to yield approximately 1 X 10^{10} organisms per ml. The two-way cocktail was made by taking 5 ml of each *L. paracasei* subspecies *paracasei* and *B. ovatus* (as described above) and mixing them together in a sterile serum vial. The three-way cocktail was prepared by mixing 5 ml a *Streptococcus alactolyticus* ATCC # PTA-4094 culture to the two-way cocktail. An overnight culture of *S. alactolyticus* was used to inoculate a 50 ml bottle of Brain Heart Infusion broth (Becton Dickson, Difco, USA) and allowed to incubate for 24 hours at 37° C. Following incubation, the inoculum was titrated on BAP and found to contain approximately 1 X 10^9 CFU per ml.

Inoculum was passed into the sterile isolators and 1 ml of the respective cultures were administered orally to each animal, at 2 days of age.

Salmonella. *S.* Typhimurium strain HL 10969pp was derived from a naladixic acid resistant strain χ4232, and genetically modified to produce green florescent protein by Chris Baum using previously described methods (2).

Challenge and necropsy. The *Salmonella* inoculum was prepared to yield approximately 5 X 10^9 organisms per ml as previously described (25). Each pig was intranasally inoculated with 0.5 ml of inoculum per naris. Three hours after inoculation the animals were humanely euthanized with SleepAway (Fort Dodge Animal Health, USA) and 3 to 5 g of the following tissues were taken for culture: blood, tonsil, mandibular lymph node, thymus, lung, liver, ileocecal lymph node, colon contents, cecum contents, diaphragmatic muscle, kidney, and ileum.

***Salmonella* culture.** Tissue samples for qualitative analysis were collected and processed as previously described (25). Qualitative samples were recorded as to the presence or absence of *S.* Typhimurium HL 10969pp. For quantitative analysis tonsil, cecum content, ileocecal lymph node (trial 1), and colon content (trial 2) samples were bluntly macerated with a rubber mallet. Following maceration all samples were serially diluted 10-fold in phosphate buffered saline (PBS) and directly plated on Xylose Lysine Deoxycholate agar (Fisher Scientific, USA) containing 50 μg of naladixic acid per ml of medium. Plates were then incubated at 37° C for 24 hours at which time colonies typical of HL 10969pp were counted and the number of *Salmonella* determined in each tissue.

Results

Environmental room swabs, rectal swabs, and pooled pen fecal samples collected from the conventional control animals and their housing facilities were negative for *Salmonella* prior to the study's initiation. Throughout the duration of the study, all rectal and skin swabs of germfree animals remained free of bacterial contamination.

The dissemination of ASI to alimentary and non-alimentary tissues with *S.* Typhimurium HL 10969pp in conventional control pigs, germfree pigs, and pigs receiving the various probiotic cocktails can be found in table 1 and table 2, respectively.

Discussion

It has been previously shown that prevalent swine-associated *Salmonella* serovars are capable of acutely infecting alimentary and non-alimentary tissues of conventional pigs within 3 hours of intranasal inoculation (25). Though acute infection has not been studied in germfree swine, it has been previously reported that germfree pigs can are highly susceptible to infection by virulent *S.* Typhimurium as indicated by dehydration, diarrhea, and septicemia (3, 12, 14, 23).

The study reported here found that acute *Salmonella* infection occured germfree pigs. *S.* Typhimurium strain HL 10969pp were capable of acutely infecting both alimentary and non-alimentary tissues of germfree pigs, conventional control pigs, and pigs receiving various probiotic treatments within 3 hours of intranasal inoculation.

Salmonella were isolated in similar percentages from conventional control pigs, germfree pigs, pigs inoculated with *L. paracasei*, and pigs inoculated with *B. ovatus*. This study suggests that pigs receiving the 2-way (*B. ovatus and L. paracasei*) and 3-way (*B. ovatus, L. paracasei,* and *S. alactolyticus*) cocktails were capable of reducing the number of tissues infected with *Salmonella* and the number of *Salmonella* isolated from alimentary and non-alimentary tissues 3 hours following inoculation. Though individual probiotic organisms were capable of reducing the number of *Salmonella* isolated from alimentary tissues their presence actually increased the number of alimentary tissues that became *Salmonella* positive.

Though the probiotics appear to be effective in reducing ASI the data presented here should be considered preliminary due to the small numbers of pigs used in these trials. This study established a probiotic model for reducing acute *Salmonella* infection and suggests that further research on the ability of the 2 and 3-way cocktails should be evaluated in their abilities to reduce ASI in conventional pigs.

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Table 1. Qualitative *Salmonella* isolation from pigs receiving various treatments

	Conventional controls	Germfree	*Bacteroides ovatus*	*Lactobacillus paracasei*	2-way cocktail[a]	3-way cocktail[b]
Number of pigs	4	5	4	3	4	2
Alimentary Tissue Totals						
Tonsil	4	5	4	3	4	2
Ileum	3	5	4	3	4	1
Colon Conts	4	2	3	3	2	0
Cecum Contents	2	5	3	3	1	0
% Positive	**81**	**85**	**88**	**100**	**69**	**38**
Non-alimentary Tissue Totals						
Mandibular lymph node	3	4	4	3	2	0
Thymus	4	4	4	2	3	2
Lung	4	5	4	3	3	1
Liver	4	4	4	3	2	0
Spleen	4	5	3	2	2	0
Ileocecal lymph node	4	3	3	3	3	1
Kidney	3	3	3	2	1	0
Muscle	3	5	2	0	2	1
Blood	2	4	3	3	2	0
% Positive	**86**	**82**	**83**	**77**	**56**	**28**
% Positive (All Tissues)	**85**	**83**	**85**	**85**	**60**	**31**

[a] 2-way cocktail consists of *Bacterioides ovatus* and *Lactobacillus paracasei*

[b] 3-way cocktail consists of *Bacteroides ovatus, Streptococcus alactolyticus*, and *Lactobacillus paracasei*

Table 2. Number of *Salmonella* (organisms per gram) isolated from pigs receiving various treatments

	Conventional controls	Germfree	*Bacteroides ovatus*	*Lactobacillus paracasei*	2-way cocktail[a]	3-way cocktail[b]
Tonsil	1.3×10^4	6.3×10^6	2.6×10^6	9.8×10^6	4.2×10^6	3.1×10^3
Colon Conts	1.7×10^3	2.2×10^4	7.8×10^2	3.3×10^0	1.4×10^3	1.4×10^3
Cecum Contents	1.5×10^5	2.1×10^6	4.8×10^3	2.4×10^2	$3.3\ 10^4$	0
Ileocecal lymph node	1.2×10^1	1.2×10^1	5.3×10^4	ND	1.2×10^1	5×10^0

[ND] Not Done

[a] 2-way cocktail consists of *Bacterioides ovatus* and *Lactobacillus paracasei*

[b] 3-way cocktail consists of *Bacteroides ovatus, Streptococcus alactolyticus*, and *Lactobacillus paracasei*

CHAPTER 6: CONCLUSIONS

Salmonella continues to be a major cause of disease in both humans and animals. The observation that *Salmonella* can acutely infect pigs within 3 hours after inoculation highlights concerns that acute *Salmonella* infection may occur in pigs during transportation to slaughterhouses and during lairage immediately prior to slaughter. Multiple manuscripts concerning naturally occurring acute *Salmonella* infection have been published, but insight into acute *Salmonella* infection in laboratory settings has not been investigated. The overall purpose of this dissertation was to better understand acute *Salmonella* infection in pigs under experimental conditions.

This dissertation identified that various virulent, avirulent, and mutant *Salmonella* isolates and strains are capable of causing varying degrees of acute *Salmonella* infection in swine. An intranasal minimal infectious dose and infectious dose 50 calculations were determined for acute *Salmonella* infection. An environmental model of acute *Salmonella* infection was established and utilized to determine *Salmonella* levels needed to cause acute *Salmonella* infection in pigs. A germfree acute *Salmonella* infection model was established and indicated that germfree piglets are capable of becoming acutely infected by *Salmonella*. Finally, probiotics were utilized in attempt to reduce or eliminate acute *Salmonella* infection. A 3-way probiotic cocktail was developed that provided partial protection from acute *Salmonella* infection in gnotobiotic pigs.

Future research directions should focus on determining the mechanism behind acute *Salmonella* infection and evaluation of intervention methods to reduce and eliminate acute *Salmonella* infection. More specifically, researchers in the United States should evaluate the effectiveness of the Danish *Salmonella* reduction model or a modification of the Danish

model. This model should focus on monitoring the levels of *Salmonella* on individual farms, categorizing farms based on herd *Salmonella* prevalence, requiring separate lairage of pigs from differing *Salmonella* categories, and mandating disinfection of lairage between groups of pigs.

APPENDIX A: EVALUATION OF A DIET FREE OF ANIMAL PROTEIN IN GERMFREE SWINE

A paper published in *Xenotransplantation* 12(2):149-155

A.T. Loynachan, J.E. Pettigrew, B.S. Wiseman, R.A. Kunkle, and D.L. Harris

Introduction

Basic research into xenotransplantation has made remarkable strides to identify and overcome potential immunological and xenozoonotic obstacles (3, 8, 15, 22, 23, 24). Concerns regarding the transmission of potential xenozoonotic agents, such as porcine endogenous retroviruses (PoERVs), still exist, but recent discoveries regarding PoERVs in pigs is aiding in the solution of the problem by clarifying PoERVs mode of transmission and allowing for risk assessment (12). Strategies for the control and elimination of PoERVs and other potential xenozoonotic agents to prevent the infection of pigs whose organs may be used for xenotransplantation (organ-source pigs) revolve around the establishment of designated pathogen-free swine colonies (DPF) that require pigs to be fed diets free of all animal protein (13).

DPF colonies have been created to establish disease-free breeding stock for xenotransplantation organ-source animal production (16). Maximum biosecurity facilities in which DPF colonies are housed limit the exposure of the DPF founder populations to potential pig and human pathogens. Onions *et al.* has suggested hysterectomy or segregated early weaning (Isowean) for DPF breeding stock producing organ-source pigs to eliminate transmission of possible pathogens from the DPF breeding stock to the organ-source animals (13). Following birth, these piglets would be placed under barrier isolation, such as that commonly used for rearing high-health and immunocompromised laboratory animals, and

fed a strict vegetarian diet without animal proteins or any bovine products, except milk-derived lactose.

Animal feed is an important potential entry point of pathogens onto a farm (4, 5). Feed-associated pathogens can pose health concerns directly for the animal population, or for the public by eventually contaminating human food or tissues that may be utilized for xenotransplantation (4). Since the discovery of prion agents capable of causing transmissible spongiform encephalopathies, concerns regarding the transmission of prions by consumable products and xenotransplantation have surfaced (1, 9). Sterilization or irradiation of feed can eliminate potential fungal, bacterial, and viral pathogens from infecting animals, but prion proteins have been demonstrated to withstand conventional autoclave sterilization and irradiation (2, 7).

In laboratory settings, orally-fed abnormal prion-infected central nervous (CNS) tissue material capable of causing bovine spongiform encephalopathy (BSE) in cattle has transmitted the prion to a number of non-bovine species (25). Recent studies have indicated that feeding 1.2 kg of prion-containing bovine CNS to swine failed to transmit BSE over a six year observation period (25). Though natural or experimental oral transmission of spongiform encephalopathies has not been recorded in pigs, experimental BSE prion infection in pigs has been reported following intracerebellar inoculation, indicating the potential susceptibility of pigs to prion infections remains an issue (25). Concerns about the transmission of prion proteins in feed have led to the development of a diet free of animal protein (DFAP).

The ability of piglets to grow and develop properly can be greatly influenced by the piglets' established microflora and the diet they are fed (10, 11). The production of

pathogen-free organ-source pigs by hysterotomy or segregated early weaning may limit the proper establishment of endogenous porcine microflora. The ability of the piglets to survive on a DFAP mandates that the diet be simple, nutritious, easily digested, and absorbed. A DFAP has not been previously developed for neonatal piglets, and the addition of characterized probiotic organisms may provide bacterial enzymes that aid in the availability and digestibility of the diet, increasing animal growth and well being (6).

Objectives of this study were to evaluate the ability of germfree piglets and *Lactobacillus paracasei* monoassociated piglets to survive and grow on a diet free of animal protein as compared to a traditional milk-based diet.

Materials and Methods

Experimental animals. Two pregnant crossbred sows of 111 days gestation were obtained from a farm located in the midwestern United States. Sows were transported to the National Animal Disease Center in Ames, Iowa where at 112 days of gestation non-survival hysterotomies were performed producing germ-free piglets (19). Sows were anaesthetized by utilizing 7% halothane for induction and maintenance. Following hysterotomy the piglets were placed into sterile transfer isolators and randomly assigned to one of four principal treatment groups. The transfer isolators were attached to sterile housing isolators and the piglets were transferred into the housing isolators that had been previously sterilized with 10% ethylene oxide for 20 hours, allowed to vent for 48 hours, and then warmed to 31° C for piglet comfort (21). Skin and rectal swabs were taken every 4 to 5 days to verify that the isolators remained free of bacterial contamination, and animals were observed daily as to general disposition (attitude, hair coat, appetite, and fecal consistency).

Experimental design. Two separate trials were conducted in which piglets were randomly assigned to 1 of 4 treatment groups (Table 1). Piglets were fed either a commercially available milk-based diet (Esbilac) or an experimental diet free of animal protein. Two days following surgical derivation piglets were either inoculated with *L. paracasei*, or remained uninoculated. Sixteen days following surgical derivation piglets were humanely euthanized and selected organs were cultured for the presence of *L. paracasei.*

Bacterial strain. *Lactobacillus paracasei* subspecies *paracasei* isolate GS-1 was previously isolated from the gastrointestinal tract of a healthy pig by methods described by Robinson *et al.* and identified with the use of API 50 CH carbohydrate metabolism strips (BioMerieux, USA) (17).

Probiotic inoculation. *L. paracasei* subspecies *paracasei* inoculum was prepared by inoculating 0.5 ml of an overnight culture into a 50 ml bottle of Bacto® Lactobacillus MRS Broth (MRS) (Fisher Healthcare, USA). The 50 ml MRS bottle was incubated for 24 hours at 37° C. Inoculum doses of *L. paracasei* subspecies *paracasei* were 1.7×10^9 and 1.8×10^9 for trials 1 and 2, respectively. Inoculum was passed into the sterile isolators and 1 ml was administered orally to each animal, at 2 days of age.

Diets. Pigs were given either an experimental diet free of animal protein (DFAP) based on soy protein, lactose, vegetable oil, minerals and vitamins (diet formulation not provided due to a pending patent), or a traditional milk-based diet (Esbilac; Pet Ag, USA). The experimental diet was pasteurized, homogenized, and irradiated. Each pig was fed individually 3 times a day for a total of 360 ml of its respective diet for the first 7 days. On day 8 each animal was fed 2 times a day for a total of 400 ml of its respective diet. The total

amount fed each pig increased by 20 ml on subsequent days until each animal received a total of 500 ml of diet daily.

Necropsy. On day 16 (14 days post-probiotic inoculation) pigs were necropsied and, in order to determine establishment and dissemination of *L. paracasei* subspecies *paracasei,* the following tissues were taken for culture: mandibular , ileocecal and jejunal lymph nodes, tonsil, thymus, lung, liver, spleen, kidney, muscle, blood, ileum, stomach contents, colon contents, and cecum contents. Swabs of the skin, tricuspid valve, and nasal turbinates were taken.

Culture. Ileocecal lymph node (trial 1), colon content (trial 2), tonsil (trials 1 and 2) and cecum content (trials 1 and 2) samples were quantitatively analyzed by macerating the tissue sample with a rubber mallet and plating serial dilutions onto MRS plates (Becton-Dickson, Difco). The ileocecal lymph node was quantitatively analyzed in trial 1, but due to lack of isolation from this tissue, the colon was chosen for quantitative analysis in trial 2. Additionally, the undiluted macerated sample was aseptically swabbed with sterile cotton tipped applicators (Fisher Healthcare, USA) and directly plated onto two sets of 5% Columbia sheep blood agar plates (BA) (Remel, USA) to check for isolator contamination. MRS plates and one set of BA plates were incubated anaerobically under 10% hydrogen, 5 % carbon dioxide, and balanced with nitrogen gas (Proxair, USA) in a Bugbox™ anaerobic work station (Toucan Technologies, United Kingdom) for 24 hours at 37° C. The second set of BA plates was incubated aerobically for 24 hours at 37° C. The remaining tissue samples were macerated with a rubber mallet, aseptically swabbed with a sterile cotton swab, and directly plated onto MRS, aerobic BA plates, and anaerobic BA plates for qualitative isolation of *L. paracasei* and identification of isolator contamination, respectively. Skin,

tricuspid valve, and nasal turbinate swabs were directly plated onto MRS plates and two sets of BA plates for qualitative analysis. MRS plates and one set of BA plates were incubated anaerobically for 24 hours at 37° C. The second set of BA plates was incubated aerobically for 24 hours at 37° C.

Statistics. Data from trials 1 and 2 were combined and examined using multiple analysis of variance methodology with JMP statistical software version 5.0 (SAS Institute Inc., USA).

Results

In trials 1 and 2, rectal and skin samples of germfree pigs (pigs A-B, E-F, I-K, and O-Q) remained free of bacterial growth throughout the duration of the trial. *L. paracasei* monoassociated piglets (pigs C-D, G-H, L-N, and R-T) also remained free of bacterial growth until probiotic inoculation on day 2. All rectal and skin swabs of monoassociated pigs following day 2 were found to be positive based on the presence of bacterial colonies representative of *L. paracasei* on MRS, aerobic BA, and anaerobic BA plates. During the duration of the study no microbial contaminants were isolated.

General pig disposition remained consistent among all treatment groups and between trials, except for animals E and F in trial 1. Thirteen days after surgical derivation in trial 1 animals E and F developed loose stools and a rough hair coat. Those animals continued to eat, but the loose stools remained until the end of the trial. Overall, pigs between groups and among trials retained appetite, remained active, alert and vocal, and produced formed feces.

All pigs on both the Esbilac and experimental (DFAP) diets remained viable for the duration of the study. Germfree pigs given the Esbilac diet gained on average a total weight of 1034 ($\pm$ 63.0) g and had a feed conversion ratio of 0.17 ($\pm$ 0.01) g of gain per 1 ml of diet

(see table 2 and charts 1 and 2). Germfree pigs fed the experimental diet gained on average a total weight of 599 (± 151) g and had a feed conversion ratio of 0.10 (± 0.02) g of gain per 1 ml of diet.

Pigs monoassociated with *L. paracasei* and fed an Esbilac diet gained on average a total of 862 (± 70.3) g and had a feed conversion ratio of 0.14 (± 0.01) g of gain per 1 ml of diet. Pigs monoassociated with *L. paracasei* and fed the experimental diet gained on average a total weight of 563 (± 96.8) g and had a feed conversion ratio of 0.09 (± 0.02) g of gain per 1 ml of diet.

L. paracasei established similarly irrespective of the diet the pigs were fed (see table 3). In animals fed the Esbilac diet, *L. paracasei* was isolated in varying degrees from all tissues except the liver and heart valve. In the alimentary tract, *L. paracasei* established to levels of, on average, 8.67×10^6, 1.81×10^4, and 1.04×10^8 organisms per gram of tissue in the tonsil, colon contents, and cecum contents, respectively. Similarly, in animals fed the experimental diet, *L. paracasei* was isolated from all tissues except the heart valve, ileocecal lymph node, and the stomach contents. In animals fed the experimental diet, *L. paracasei* reached, on average, 7.25×10^6, 2.47×10^4, and 7.4×10^8 organisms per gram of tissue in the tonsil, colon contents, and cecum contents, respectively.

Statistical differences ($p>0.05$) were not seen in initial pig weights at day 0. *L. paracasei* had no effect ($p>0.05$) on piglet growth, as measured by total weight gain, and feed conversion. There were also no *L. paracasei* by diet interactions.

Statistical differences ($p<0.05$) were noted between trial 1 and 2 on measured growth parameters of pigs receiving the experimental diet without *L. paracasei*. Additionally, the diet affected the measured growth parameters.

Discussion

As research pertaining to xenotransplantation advances, the prospect of utilizing animal tissues and organs in humans may one day become reality. With this advancement, concerns regarding organ contamination with prions and other infectious agents have been expressed (9). For this reason animals used in xenotransplantation research have been reared under barrier isolation, greatly reducing the opportunities for both pig and human pathogens to enter the transplantation organ production chain (16). Though barrier isolation is highly effective at keeping pathogens out, it may also effectively keep beneficial natural microflora from establishing. Barrier-reared pigs have been fed animal-based feeds for reasons of development and growth, but concerns of prion transmission to tissue recipients may ultimately end this practice. The research presented here provides an evaluation of a novel experimental diet capable of supporting life in germfree pigs.

Pigs fed the experimental diet, excluding two animals which developed mild diarrhea, lived comparably to pigs fed the Esbilac diet, although they grew more slowly. Advantages of milk proteins in the diet of artificially reared pigs had been shown previously.(14) Both diets were effective in producing viable pigs, and we suggest this result would have continued beyond the short 16-day term of this experiment. Practical recommendations for feeding young pigs emphasize reductions in the levels of milk products and other animal-source ingredients as the pigs age and their digestive and immune systems mature, suggesting that the importance of the milk proteins would decline with increasing age of the pigs (20). Additional support for this conclusion comes from the widespread practice of weaning pigs near 16 days of age.

When comparing the two diets, animals fed the Esbilac diet gained more weight, and thus had higher average daily gains and higher feed conversion ratios compared to piglets reared on the experimental diet. This deficit in the experimental diet is most likely due to the lack of specific physiologically active components of milk, or of specific mammalian and bacterial enzymes needed to degrade the experimental diet into utilizable nutrients (6). The addition of the bacterium *Lactobacillus paracasei* subspecies *paracasei* did not readily enhance utilization of the experimental diet.

L. paracasei extensively established to similar levels in both alimentary and non-alimentary tissues irrespective of the diet the animals were fed. *L. paracasei* had no effect on growth in animals fed either the Esbilac or experimental diets. *L. paracasei* may have provided bacterial enzymes that could have helped the animals utilize the differing diets nutrients, but the bacteria may have also been effectively utilizing more nutrients for its own growth and replication or stimulating costly inflammatory reactions in the intestinal tissues, thus limiting nutrient availability to the pigs. Additionally, due to the short duration of the study and the requirement that the animals be fed a liquid diet, any long term beneficial effects due to *L. paracasei* colonization or beneficial effects in animals reared on a solid diet may have been overlooked. Though *L. paracasei* did not increase feed efficiency, data are still inconclusive as to whether the addition of other untested beneficial bacteria may increase the amount of available nutrients in the experimental diet. Individual trial variance within the principal group receiving the experimental diet without *L. paracasei* can not be directly explained, but can be attributed to the poor weight gain in animals E and F in trial 1. An undetected difference in germfree isolator environments in trial l may have caused these two animals to have diarrhea. Though statistical analysis was preformed the small number of

pigs used in these studies, due to the high cost and intensive labor involved in rearing germfree and gnotobiotic swine, may have hindered the interpretation of the statistical analysis.

Though lactic acid bacteria, such as *Lactobacillus sp.*, are categorized as generally safe (GRAS), some species of this genus are capable of regionally infecting the host and ultimately entering the host's blood (18). Organisms, including *L. paracasei*, capable of dissemination to and colonization of non-alimentary tissues, may eventually cause an unwanted disease state in these DPF pigs. This finding may ultimately indicate that this organism is a poor probiotic choice in pigs whose organs may be utilized in humans.

In summary, an experimental diet free of animal protein has been developed and evaluated in both germfree and gnotobiotic pigs. Our data suggests that the experimental diet is capable of sustaining life, but is not as efficacious as conventional milk-based diets in total weight gain and feed conversion. The additional establishment of the potential probiotic bacterium *L. paracasei* did not benefit piglet growth when given in conjunction with either a milk-based or experimental diet.

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Chart 1. *L. paracasei* effect on total weight gain for each diet.

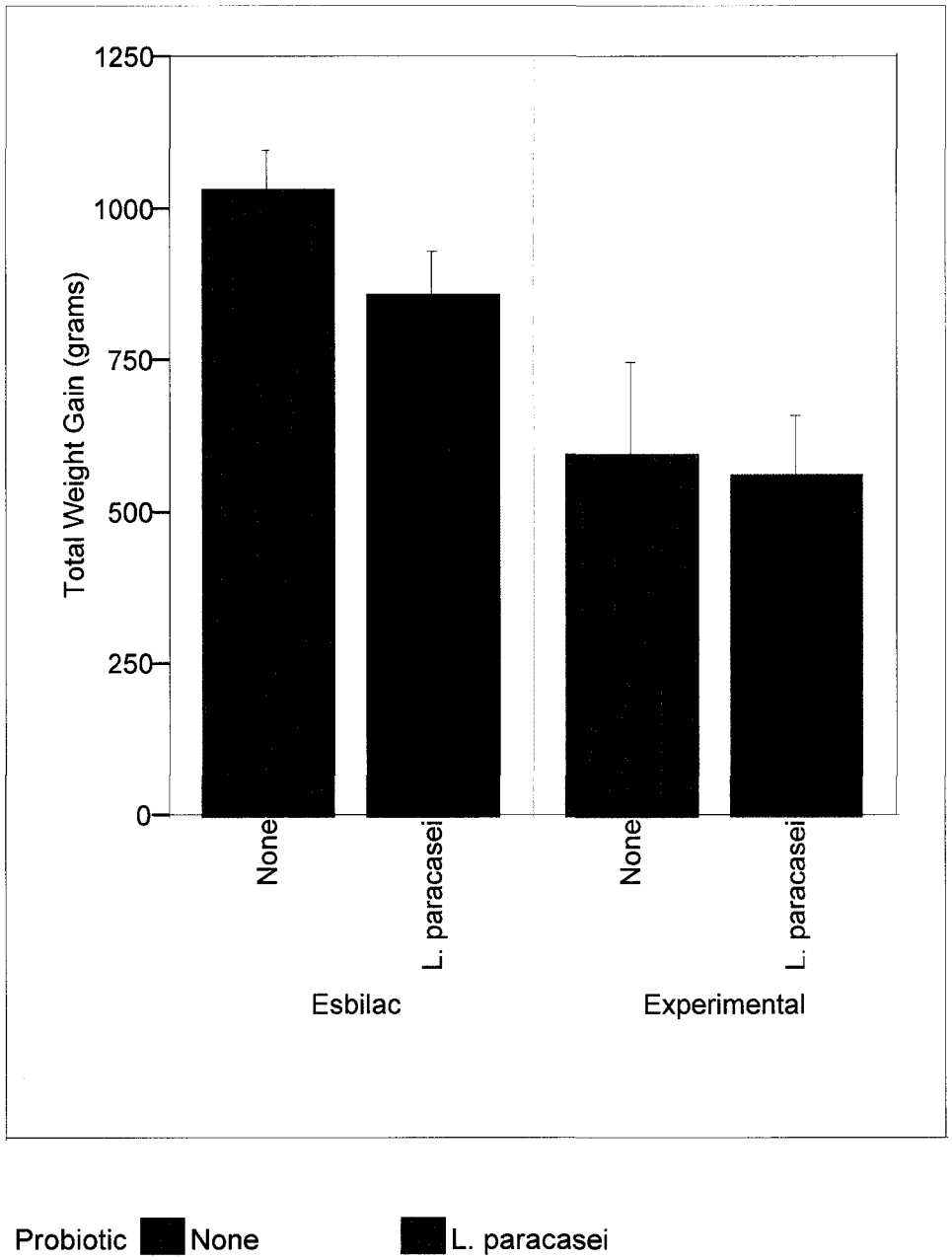

Chart 2. *L. paracasei* effect on feed conversion for each diet

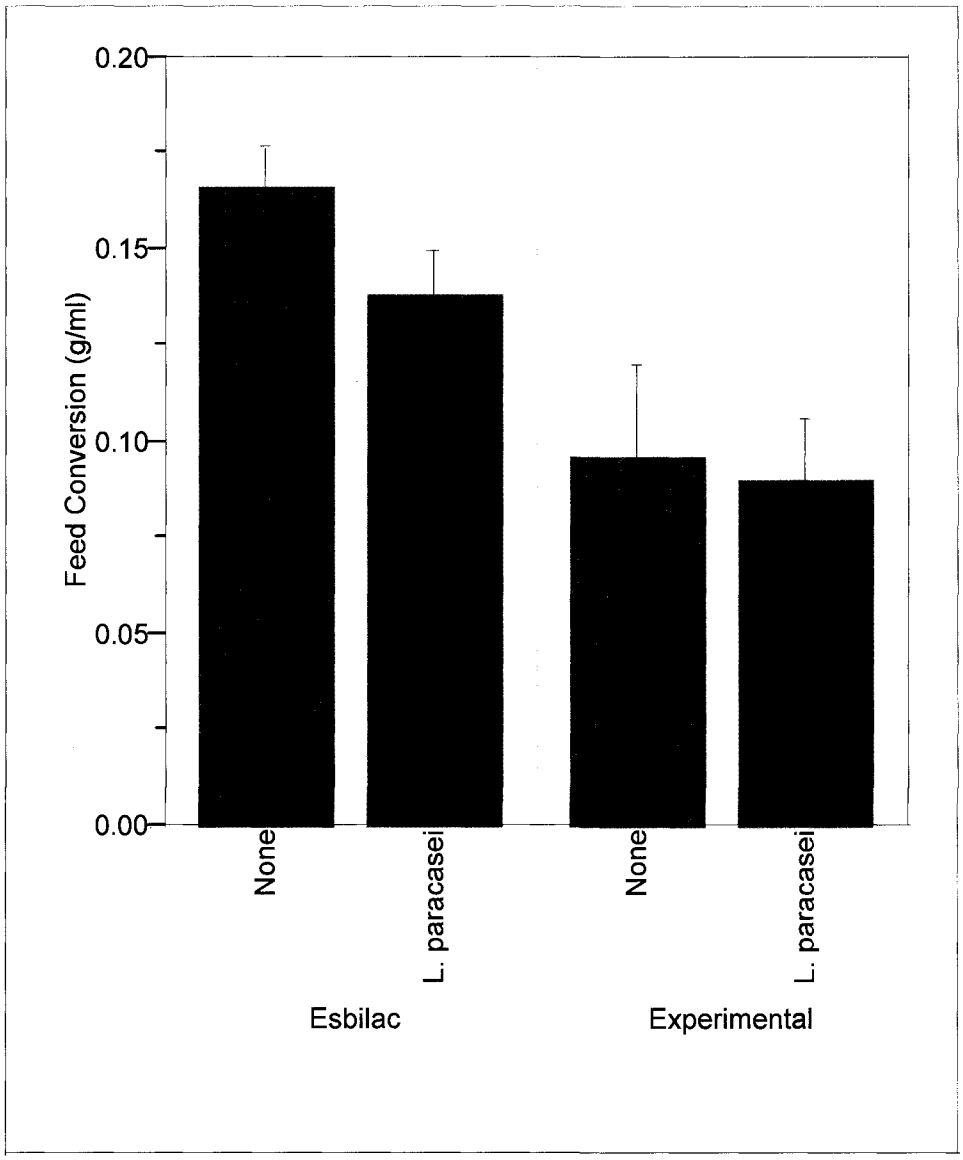

Table 1. Experimental design

Trial	Number of Animals	Diet	Monoassociation with *L. paracasei*
1	2	Esbilac	No
	2	Esbilac	Yes
	2	DFAP	No
	2	DFAP	Yes
2	3	Esbilac	No
	3	Esbilac	Yes
	3	DFAP	No
	3	DFAP	Yes

Table 2. Total weight gain, average daily gain, and feed conversion for each animal.

Trial	Animal ID	Diet	Probiotic	Total weight gain	Feed conversion
1	A	Esbilac	none	862	0.139
	B	Esbilac	none	953	0.153
	C	Esbilac	L. paracasei	953	0.153
	D	Esbilac	L. paracasei	1089	0.175
	E	Experimental	none	408	0.066
	F	Experimental	none	136	0.022
	G	Experimental	L. paracasei	454	0.073
	H	Experimental	L. paracasei	454	0.073
2	I	Esbilac	none	1179	0.19
	J	Esbilac	none	1179	0.19
	K	Esbilac	none	998	0.16
	L	Esbilac	L. paracasei	726	0.117
	M	Esbilac	L. paracasei	817	0.131
	N	Esbilac	L. paracasei	726	0.117
	O	Experimental	none	998	0.16
	P	Experimental	none	816	0.131
	Q	Experimental	none	635	0.102
	R	Experimental	L. paracasei	363	0.058
	S	Experimental	L. paracasei	907	0.146
	T	Experimental	L. paracasei	635	0.102

Table 3. *L. paracasei* establishment for each tissue (trial 1 and 2 combined)

n=5	**Esbilac**	**Experimental**
Skin	2	4
Rectum	5	5
Blood	2	1
MLN	2	2
Lung	1	1
Heart Valve	0	0
Liver	0	1
Spleen	2	1
Kidney	2	1
Jejuninal Ln.	2	2
ICLN	1	0
Ileum	3	5
Stomach Contents	3	0
Tonsil	5	5
quantitative counts	8.67×10^6	7.25×10^6
Colon Content	5	5
quantitative counts	1.81×10^4	2.47×10^4
Cecum Contents	5	5
quantitative counts	1.04×10^8	7.4×10^8

APPENDIX B: PROBIOTIC COMPOSITIONS AND METHODS AGAINST BACTERIAL INFECTION IN LIVESTOCK ANIMALS

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On 16 October 2003 the authors and Iowa State University filed an international patent (ISURF # 02757; I9000.0050/P050) for the reduction of bacterial infections by use of varying probiotic compositions. Currently, the patent disclosure is still in the approval process under the council of the Disckstein Shapiro Morin & Oshinsky LLP law firm in Washington, DC.